A potential treatment for insomnia by positive allosteric modulation of adenosine A2A receptors

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List of Abbreviations

ADA: Adenosine deaminase
AMP: Adenosine monophosphate
ATP: Adenosine triphosphate
BDZ: Benzodiazepine
BBB: Blood brain barrier
BF: Basal forebrain
cAMP: Cyclic adenosine monophosphate
CHO: Chinese hamster ovary
DAT: Dopamine transporter
DMEM: Dulbecco's Modified Eagle's Medium
DREAAD: Designer receptor exclusively activated by designer drugs
EEG: Electroencephalography
EMG: Electromyography
FBS: Fetal bovine serum
FRET: Forster resonance energy transfer
GABA: Gama amino butyric acid
HBSS: Hank's Balanced Salt Solution
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IBMX: 3-isobutyl-1-methylxanthine
ICV: Intracerebroventricular
IP: Intraperitoneal
KO: Knockout
NAc: Nucleus accumbens
NEAA: Nonessential amino acid
PAM: Positive allosteric modulator
QPCR: Quantitative polymerase chain reaction
REM sleep: Rapid eye movement sleep
RNA: Ribonucleic acid
SWS: Slow wave sleep
TMN: Tuberomammillary nucleus
VLPO: Ventrolateral preoptic nucleus

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Introduction

I.1 A general view of sleep substances

Sleep is an intensive anabolic state when bodies are carrying outgrowth and repair processes such as the formation of new bones, muscular, and nervous tissues. Most animals, including humans and other mammals, birds, a significant number of reptiles, amphibians, and fish do sleep (Vorster and Born, 2015). Sleep-wake cycle is known for regulating by two biological mechanisms in the body, which has a link and balance each other. This model is also cited as the two-process model of sleep-wake regulation. Circadian rhythm often referred to as Process C, controls the body's internal processes and alertness levels, which is run by the internal biological or circadian clock. Sleep-wake homeostasis, also known as Process S, is the generation of a homeostatic sleep drive by the accumulation of hypnogenic (sleep-inducing) substance in the brain (Borbély, 1982). Source of the need for sleep often referred, as "sleep drive" mainly remains uncertain in the neural and cellular basis. But it can be considered that a homeostatic pressure, which dissipates during sleep, builds up during the wake period. In 1892, Rosenbaum, at the first time, reported that the role of humoral factors in the regulation of sleep (Rosenbaum, 1892). Following this first report, many researchers independently showed that the existence of sleep-promoting chemicals. Over the past century, several additional hypnogenic substances involved in the sleep homeostatic process have been identified including prostaglandin D2 (Qu et al., 2006a; Ueno et al., 1982), cytokines (Krueger et al., 1984a), adenosine (Porkka-Heiskanen et al., 1997), anandamide (García-García et al., 2009a), and the urotensin II peptide (Huitron-Resendiz et al., 2005).

I.2 The role of adenosine in physiological systems

Adenosine is a pervasive nucleoside that contributes to building blocks of the energy storage molecule, ATP and nucleic acids as well as a substrate for multiple enzymes, and most importantly is an extracellular modulator of cellular activity (Illes et al., 2000). Adenosine has been widely studied in various tissues after its first description in 1929 by Drury and Szent-Gyorgyi (Drury and Szent-Györgyi, 1929) There is a wide range of organ systems affected by the endogenous release of adenosine (Olah and Stiles, 1992). For instance, adenosine has an essential hyperpolarizing effect on the membrane potential of excitable cells, play an inhibition role in vascular smooth muscle cells of coronary arteries and neurons in the brain. Adenosine mostly plays an inhibitory neuromodulator role, not a neurotransmitter. It is inhibiting the neuronal activity of excitatory neurons (e.g. glutamatergic and cholinergic) as well as inhibitory neurons (e.g. GABAergic). Adenosine increases potassium conductances while postsynaptically inhibiting neuronal activity(Porkka-Heiskanen et al., 2002). The formation of adenosine can be increased by numerous cellular process and in all parts of these cells (Rudolphi et al., 1992; Fredholm, 1997; Ongini and Schubert, 1998; Von Lubitz, 1999; Latini and Pedata, 2001). Physiological level of adenosine relies on fundamental cell biology and independent neuron activation. Extracellular accumulation of adenosine highly correlated with the ration of oxygen decreases in the condition like hypoxia, ischemia, and hypo-perfusion (Winn et al., 1980; Van Wylen et al., 1986). Therefore, it has been considered as a self-controlling mechanism of the cells in order to minimize the cell damage. It has been also suggested that extracellular adenosine level start to increase in response to prolonged

neuronal activity during prolonged wakefulness period in the related brain area (i.e. basal forebrain). Thus, sleep induced in order to keep energy balance of the brain before it affected. In conclusion, it hypothesized that local energy reduction in the basal forebrain area will induce increased extracellular adenosine level and sleep (Porkka-Heiskanen et al., 2002).

I.2.1 Adenosine level and its regulation

Adenosine monophosphate (AMP) and S-adenosylhomocysteine are the two well-known molecules that play the role in the formation of adenosine (Fredholm, 2007; Schrader, 1983). The activity of the enzyme S-adenosylhomocysteine hydrolase on S-adenosylhomocysteine forms adenosine. It can also act to snare adenosine in the existence of excess L- homocysteine. This process appears intracellular level and it is the way that the enzyme bidirectional ensures the persistent presence of a finite concentration of adenosine in the cell. The intracellular adenosine kinase generates AMP from adenosine, which can generate by 5' nucleotidase. This mutual activity keeps the constant presence of a predetermined intracellular level of adenosine in the range of 10 to a few hundred nanomolar (nM) under physiologic circumstances (Ballarín et al., 1991).

Since all cells appear to possess one or more equilibrative purine transporters, the predetermined intracellular concentration of adenosine ensures that there is also a not insufficient extracellular concentration of adenosine. Releasing of adenine nucleotide is the main case for the increasing level of extracellular adenosine (Geiger and Fyda, 1991). The series of ectoenzymes on the cell surface forms extracellular adenosine by the conversion of ATP to adenosine. It has been reported that many cell types can release

extracellular ATP by various mechanisms, including co-release from storage vesicles together with other neurotransmitters, lysosome exocytosis, a “kiss and run” mechanism (MacDonald et al., 2006), release from inflammatory cells or vascular endothelial via connexin hemichannels and channels such as P2X7 receptors (Chen et al., 2006; Faigle et al., 2008; Linden, 2006), unrestrained leakage from necrotic cells (Eltzschig, 2009), and controlled release through pannexin hemichannels (Chekeni et al., 2010; Elliott et al., 2009). AMP forms by breaking down of extracellular ATP and adenosine diphosphate (ADP) via multiple ectoenzymes, exclusively CD39 (Yegutkin, 2008). Ecto-5’-nucleotidase, CD73 broken down formed AMP to adenosine is the only way in the brain(Resta et al., 1998).

Essentially, the cell membrane breaking process results in an increased level of extracellular adenine. Therefore, any form of the trauma is connected to elevate the level of adenine nucleotides. In addition, cellular apoptosis also results in direct releasing of AMP (Yamaguchi et al., 2014).

Endothelial cells, astrocytes, and neurons are the reported sources for the releasing of ATP via many different mechanisms (Bodin and Burnstock, 1998; Guthrie et al., 1999; Fields and Stevens, 2000). It is hypothesized that extracellular adenosine originates from neurons and from adjacent non-neuronal cells like glial cells in the brain (Halassa et al., 2007, 2009). For instance, it proposed that astrocytes are an important source of extracellular adenosine via gliotransmission (Halassa et al., 2009). Since CD73 enzyme degrades AMP to adenosine in the brain. It also reported that ATP released from neurons also contributes to extracellular adenosine production via the CD73 enzyme (Lovatt et al., 2012; Wall and Dale, 2013). In the striatum, the reports showed that adenosine formed

via CD73 alternatively act at the A_{2A} receptor as extracellular CD73 is selectively co-expressed and is physically linked with A_{2A} receptors in striatopallidal neurons (Ena et al., 2013; Augusto et al., 2013). Adenosine can also be arbitrated synaptic depression, which is independent of CD73 activity and not a result of neuronal or astrocytic ATP release but is because of the activation of postsynaptic neurons. Thus, it leads to a response mechanism that suppresses the excitatory transmission during prolonged activity (Lovatt et al., 2012).

It also postulated that under physiologic conditions, for instance, those involved in sleep and wakefulness, adenosine is generated independently from the CD73 activity. Adenosine deaminase (ADA) acts as decreasing adenosine level particularly when adenosine levels are high. On the other hand, adenosine kinase rapidly converts adenosine to AMP by phosphorylation in the cells. Extracellular adenosine level determines by the formation and removal process of its. These levels are low and usually in the range between 30-300 nM under physiological conditions (Ballarín et al., 1991). Adenosine level goes 1 µM to several tens of µM under extreme conditions, such as strenuous exercise or mild hypoxia, and in severely traumatic situations, including local ischemia (Fredholm, 2007).

One of the biggest challenges in today's sleep research is to link adenosine levels to sleep. It because of adenosine levels rapidly change in both the blood and tissue upon sampling and tissue samples must be frozen within a second or less to preserve in vivo levels. Such rapid inactivation makes difficult to achieve accurate measurement of adenosine level with the techniques, such as microdialysis and electrochemical methods. Therefore, reported adenosine levels in sleep and wakefulness should be carefully

accepted.

I.2.2 Adenosine Receptors

There are four subtypes of adenosine receptors have been reported. These are G protein-coupled A_1, A_{2A}, A_{2B}, and A_3 receptors (Fredholm et al., 2011). A_1R, A_3R are Gi couple receptors and activation of them inhibits the adenylyl cyclase, activates phospholipase C (PLC), opens several types of K^+ channels, and inactivates N-, P-, and Q- type Ca^{2+} channels (Gerwins and Fredholm, 1992). On the other hand, $A_{2A}R$ and $A_{2B}R$ are Gs or Golf couple receptors and activation of them increases adenylyl cyclase activity, induces the formation of inositol phosphates, and activates protein kinase C. In addition, A_1R and A_3R activation decreases cyclic adenosine monophosphate (cAMP) level while $A_{2A}R$ and $A_{2B}R$ activation increases level of cAMP (Fredholm, 1995).

While A_{2A} receptors are located mainly in the nucleus accumbens, striatum, and olfactory bulb, A_1 receptors are widely distributed in the central nervous system (Fredholm et al., 2001). On the other hand, A_{2B} receptors are also widely expressed, but generally at very low levels; whereas A_3 receptors are expressed at moderate levels in the hippocampus and cerebellum (Yaar et al., 2005). There is growing body of reports indicate that the effects of adenosine on vigilance state are mediated through A_1 and A_{2A} receptors. However, little is known about A_{2B} and A_3 receptors on vigilance state (Huang et al., 2011).

I.3 Investigating the functions of adenosine receptors

I.3.1 Uncovering functions of adenosine receptors by receptor antagonists

Selective pharmacological tools are the key point for evaluating in vivo action of the adenosine receptors. More than last two decades, agonist and antagonist molecules have been produced with high affinity and selectivity for the human variants of each of the four receptors by the medicinal chemists (Fredholm et al., 2011). While adenosine receptor antagonists have diverse structures, the most popular adenosine receptor agonists are derivative of purine nucleotides (Müller and Jacobson, 2011). There are many $A_{2A}R$ selective antagonists developed from different structural classes such as 8-(3-chlorostyryl) caffeine, MSX-2, and its water-soluble pro-dugs MSX-3, SCH-58261, KW6002, and ZM-241385. Besides that, radioactive, and more recently fluorescent, ligands of adenosine receptors were also developed and introduced for drug screening and monitoring in vivo receptor residence in humans.

Caffeine is the well-known nonselective adenosine receptor antagonist and widely consumed as an arousal-promoting psychostimulant. The daily-consumed dose of caffeine by the human produces its partial profound arousal effect at the range of 25-50% despite its weakly potent nonselective blockade of adenosine A_1 and A_{2A} receptors (Fredholm et al., 1999). Paraxanthine and theophylline, which are the metabolites of the caffeine (Arnaud, 2011), are more potent inhibitors to A_1 and A_{2A} receptors than caffeine. Thus, adenosine receptors blockade can be long lasting even caffeine elimination in the

central system.

Since A_{2A} receptors antagonist showed a very consistent and perfect safety profile in the clinical trials for Parkinson's disease (PD), this provides an insight to the pharmacological control of the sleep-wake cycle by A_{2A} receptors antagonists as well.

I.3.2 Revealing functions of adenosine receptors by receptor knockouts and other genetic techniques

Targeted deletion of specific exons on all four G-protein coupled adenosine receptors has been achieved to generate genetic knockout (KO) models. (Fredholm et al., 2004; Wei et al., 2011). On the contrary to the limitations of pharmacological control of adenosine receptors, adenosine receptors KO models provide a wide range of the information about the physiological functions of the adenosine receptors and its role on the sleep-wake cycle by targeting them in the defined cells population. For instance, A_{2A}R KO models help to the researcher for overcoming the concerns on the specificity of adenosine receptors antagonists and it clearly demonstrated that sleep-promoting effects of the A_{2A} receptors agonists and caffeine-induced arousal effects depend on the A_{2A} receptors but not A_1 receptors (Huang et al., 2005). Conditional KO of some adenosine receptors genes in specific brain regions (e.g., striatum versus forebrain) and cell-types (astrocytes versus neurons) achieved with Cre-loxP system (Fuller et al., 2015; Wei et al., 2011), provides the researcher to overcome essential limitations of global A_1 and A_{2A} receptor on confounding developmental effect and lack of cell-type specificity (Fredholm et al.,

2004). Brain region-depended deletion of the A_{2A} receptors in the forebrain (i.e. hippocampus, cortex, striatum) (Bastia et al., 2005; Yu et al., 2008) and local deletion of the A_1 receptors in CA1 or CA3 neurons and A_{2A} receptors in nucleus accumbens (NAc) has been achieved. Local injection of adeno-associated virus (AAV) which includes vectors that containing the Cre transgene into the brains of mice carrying loxP-flanked A_1 receptor (Scammell et al., 2003) or A_{2A} receptor (Lazarus et al., 2011) genes is the key method to obtain local deletion of these receptors. Conditional KO strategy revealed the role of adenosine receptors on the sleep-wake cycle in the basal ganglia. Moreover, the advancement of AAV carrying short-hairpin RNA targeted to produce site-specific silencing of the A_{2A} receptors revealed that arousal effect of caffeine is mediated by A_{2A} receptors in NAc shell (Lazarus et al., 2011). In addition, recent advancement of the optogenetics based on the modulation of the local neuronal activity by using the genetically engineered optical switches (e.g., channelrhodopsin) (Boyden et al., 2005; Deisseroth, 2014; Yizhar et al., 2011) or chemogenetics to investigate G-protein signaling in freely behaving animals by designer receptors exclusively activated by designer drugs (DREAAD) (Farrell et al., 2013; Giguere et al., 2014) has enhanced our understanding of novel brain circuits essential the sleep-wake cycle (Fuller et al., 2015). Lately, researchers also developed a probe (optoA_{2A} receptor) for specific optogenetic control of A_{2A} receptor signaling (Li et al., 2016).

I.4 Adenosine and sleep

I.4.1 Adenosine level during sleep and wakefulness

Adenosine is a well-known marker for the energy deficiency; extracellular adenosine level increase by the reduction of ATP (Kalinchuk et al., 2003)and is highly associated with the sleep promotion (Porkka-Heiskanen et al., 1997). Adenosine has been reported high level during slow-wave sleep (SWS) than wakefulness in the solution collected numerous brain area of cats by in vivo microdialysis probe while their routine sleep-wake cycle (Porkka-Heiskanen et al., 1997, 2000). In addition, in vivo microdialysis experiments demonstrated that adenosine level of the basal forebrain increase double during prolonged wakefulness in compare to the beginning of the long-lasting wakefulness (Porkka-Heiskanen et al., 1997, 2000).

Although more than a half-century past from discovery of the hypnotic effect of adenosine in mammalian brain (Feldberg and Sherwood, 1954), the brain cell types involved in sleep-inducing effects of the adenosine still not uncover. Theoretically, glial cells and neurons are the sources of the extracellular adenosine and ATP, which is quickly converted to the adenosine. In addition, genetic engineered mice studies showed that blocking ATP releases from astrocytes resulting in decreasing level of the extracellular adenosine (Pascual et al., 2005). Even though these manipulations do not change total amount of the SWS, REM, and wakefulness, they cause the reduction of slow-wave activity and rebound sleep after long-term sleep loss which is suggesting that

adenosine releasing from the astrocytes has the role on the sleep drive (Halassa et al., 2009). However, exact sources of adenosine still not elucidated.

Further reports such as the elimination of adenosine deaminase (ADA) for increasing extracellular level of adenosine in the central nervous system of rats was also confirmed REM, and SWS inducing effect of the adenosine (Radulovacki et al., 1983).

It is well described that adenosine takes the sleep modulation role via A_1 and A_{2A} receptors in the brain. However, the importance of these receptors on sleep-wake regulation remains controversial (Basheer et al., 2004; Huang et al., 2007). Early pharmacological studies with non-selective A_1/A_{2A} receptor antagonist caffeine, alloxazine, and the A_1 receptor antagonist 8-cylopenthyltheophylline showed that A_1 receptors might be more important in sleep-wake regulation than the A_{2A} receptors (Virus et al., 1990).

However, the limitations of the pharmacological studies such as differences in solubility of antagonists, neuropharmacodynamics, blood-brain barrier permeability, and most crucial “off-target” effects in a high dose of antagonists lead to the misinterpretation of data. Later reports demonstrated that A_1 receptors activation induce sleep or wakefulness in a region-specific manner (Ochiishi et al., 1999; Reppert et al., 1991; Rivkees et al., 1995). The advancement of the genetically engineering systems such as recombinant viral vectors delivery in the brain, transgenic animals, and evidence human studies clearly proof that crucial role of the A_{2A} receptors in the regulation of sleep and wakefulness over the last decade (Holst and Landolt, 2015; Lazarus et al., 2013, 2012).

I.4.2 The role of A_1 receptors in sleep/wake regulations

Early studies showed that adenosine A_1 receptors activation with the systematic or intracerebroventricular administration of A_1 receptor agonist N6-cyclopentyladenosine induced the slow-wave activity of the brain's electrical signals during SWS(Benington et al., 1995). However, the total amount of the SWS and REM sleep remained unchanged during the lateral ventricular administration of A_1 receptors antagonist (Urade et al., 2003). This suggests that A_1 receptors activation has opposite sleep-wake inducing effects in different brain regions. For instance, adenosine activity at A_1 receptors inhibiting the arousal active neurons in the basal forebrain (BF) such as substantia immoniata and horizontal limb of the diagonal band of Broca (Alam et al., 1999; Strecker et al., 2000). There are two ways reported that adenosine may promote sleep via A_1 receptors; A_1 receptors mediated inhibition of glutamatergic signal to cortically projecting GABAergic and cholinergic neurons of the BF (Yang et al., 2013) and A_1 receptors mediated suppression of orexin/hypocretin neurons in the lateral hypothalamus (Thakkar et al., 2008).

Adenosine deaminase (ADA) and histamine neurons expressing A_1 receptors are predominantly localized in the TMN region suggesting that histaminergic arousal in the TMN actively regulated by adenosine. Indeed, further reports showed that bilateral injections of A_1 receptors agonist or ADA inhibitor conformycin into the rat TMN robustly increase the amount of SWS (Oishi et al., 2008). These results demonstrate that endogenous adenosine suppresses the arousal effects of the histaminergic system via activation of A_1 receptors in TMN (Thakkar, 2011). In contrast to the sleep-inducing effect of an A_1 receptor in the TMN, activation of A_1 receptor shows the wake-promoting

effect in the lateral preoptic area of the hypothalamus (Methippara et al., 2005).

I.4.3 The role of A_{2A} receptors in the sleep/wake regulation.

Central administration of the highly selective and potent adenosine A_{2A} receptor agonist, CGS 21680 robustly increase SWS and REM sleep in mice (Satoh et al., 1996; Urade et al., 2003). Later studies demonstrated that infusions of CGS 21680 into the BF dose-dependently inhibit release of the histamine in both medial preoptic area and frontal cortex, and induce the release of GABA in the TMN of the hypothalamus, but not in the frontal cortex (Hong et al., 2005). GABA antagonist picrotoxin perfusion to the TMN blocking CGS 21680 inhibiting histamine release, which is suggesting that adenosine A_{2A} receptor agonist promote sleep by inhibiting the histaminergic system through an increase in GABA release in the TMN.

Earlier reports suggested that sleep generated by activation of sleep-promoting neurons in VLPO together with suppression of histaminergic wake-promoting neurons in the TMN through galaninergic and GABAergic inhibitory projections (Sherin et al., 1998, 1996). VLPO neurons characterized as two different cell types according to their response to serotonin and adenosine by intracellular recording in rat brain slices. An A_1 receptor agonist primarily inhibits VLPO neurons activity, which is inhibiting arousing neurotransmitters noradrenaline and acetylcholine. In addition, despite the serotonin inhibits type-1 neurons and activates type-2 neurons, adenosine A_{2A} receptors agonist postsynaptically activates type-2, but not type-1 neurons in the VLPO. These findings suggesting that type-2 neurons have an important role in sleep-inducing, however, type-1 neurons provide sleep consolidation since only the absence of inhibitory effects from the

arousal system make them activated (Gallopin et al., 2005).

The c-fos expression of neurons is not only observed in the VLPO, but also within the medial portion the olfactory tubercle and the NAc shell after the administration of CGS 21680 into the rostral BF (Satoh et al., 1999; Scammell et al., 2001). Surprisingly, direct perfusion of adenosine A_{2A} receptor agonist into the NAc promotes SWS and REM sleep as 75% amount of sleep measured when it infused into subarachnoid space. These findings suggested that A_{2A} receptors within or close to the NAc predominantly induce sleep (Satoh et al., 1999). In addition, experiments on global genetic KOs of the A_1 and A_{2A} receptors showed that arousal effects of the caffeine, non-selective adenosine receptor agonists, depend on the A_{2A} receptors but not A_1 receptors (Huang et al., 2005). Further studies by using advance site-specific gene manipulation techniques for generating conditional A_{2A}R KO mice such as silencing A_{2A}R expression via local infection with AAV carrying short-hairpin RNA of A_{2A} receptor or Cre/lox technology provides wide range of information for the understanding specific role of A_{2A} receptors in the basal ganglia (BG) (Lazarus et al., 2011). In addition, reports show that arousal effects of caffeine abolished in the absence of the A_{2A} receptors in the NAc. This result suggests that adenosine tonically activate NAc neurons in the absence of the caffeine since adenosine A_{2A} receptors abundantly express throughout the striatum, including NAc shell (Rosin et al., 1998; Svenningsson et al., 1999).

These findings also suggest that adenosine activity on A_{2A}R in the NAc suppress the arousal systems, which can be turned on with the antagonist effects of the caffeine. In addition, studies on dopamine D_2 receptors (D_2R) in the NAc revealed the opposite effect in sleep-wake regulation compare to the A_{2A}R. For instance, deletion of the dopamine

transporter (DAT), which is responsible for removing dopamine from the receptor (Giros and Caron, 1993), increases wakefulness and reduces SWS. Later studies showed that activation of A_{2A} receptors leads to deteriorating affinity of dopamine at D_2 receptors with intra-membrane interactions. Thus, adenosine and antagonist would control medium spiny projections neurons in the striatum via A_{2A} independently of D_2 receptors (Aoyama et al., 2000; Chen et al., 2001). Moreover, the studies show that the sensitivity of caffeine stimulation and homeostatic response to sleep deprivation increased in the humans who have a genetic reduction of striatal DAT (Holst and Landolt, 2015).

Adenosine-mediated modulation of sleep-wake cycle in NAc revealed the possibility of basal ganglia neurons roles in sleep-wake regulation (Lazarus et al., 2011, 2012). Broad variety of cognition, movement, and sleep-wake related disorders have link with BG dysfunction like Parkinson's and Huntington diseases as well as lesions in the BG (Adler and Thorpy, 2005; Dale et al., 2004; Goodman and Barker, 2010; Obeso et al., 2000; Wetter et al., 2000). Time spent in wakefulness together with the fragmentation of both sleep and wakefulness showed a robust reduction after bilateral lesions of the neurons in the striatum or especially in the NAc by using the ibotenic acid (Qiu et al., 2010). Lesions in the globus pallidus and caudate-putamen alone promoted wake by 50% and sleep by 10%, respectively (Qiu et al., 2010).

On the other hand, the reports showed that dopamine D_2 receptors are notably expressing in BG and complete deletion of them increases SWS and REM sleep with a dramatic decrease of SWS delta power and wakefulness (Qu et al., 2010). In addition, PD patients mainly use modafinil to treat their excessive sleepiness and reports showed that arousal effects of modafinil specifically mediated by D_1 and D_2 receptors, which makes D_2

receptors primary important (Qu et al., 2008).

These reports suggest that $A_{2A}R$ activation induced the activity of GABAergic output neurons in the striatopallidal circuits and consequently arousal network in the thalamus, brainstem, and hypothalamus and eventually the cerebral cortex are sustained tight inhibitory control. Indeed, transgenic expression of optogenetic tools channelrhodopsin and chemogenetics tools DREADD in mice via Cre-recombinase (expressed under the A_{2A} receptor promoter) dependent AAV vectors microinjections, exclusively induced SWS during specific activation of striatopallidal neurons by light or the small molecule clozapine-N-oxide (Oishi et al., 2017).

Furthermore, recent studies show that $A_{2A}R$ also have a role in REM sleep. For example, blocking $A_{2A}R$ expressing neurons in the olfactory bulb promotes REM sleep. This finding implied that the olfactory bulb is the crucial site for adenosine $A_{2A}R$-depended REM sleep regulation (Wang et al., 2017). Surprisingly, patients with the REM sleep behavior disorder have impaired smell ability (Stiasny-Kolster et al., 2007).

I.5 $A_{2A}R$ as therapeutic target

Caffeine is one of the ingredients of the daily beverages such as coffee, tea, and coke, which make it one of the most consuming compounds. Early studies show that adenosine receptors are the major targets of caffeine that later identified as a non-selective adenosine receptors antagonist. Adenosine receptors are widely expressed in the body, which provides promising therapeutic targets in a wide range of conditions, including

cerebral and cardiac ischemic diseases, immune and inflammatory disorders, cancer, and sleep disorders. Therefore, medicinal chemists show enormous efforts to produce the considerable number of selective agonists and antagonist for the adenosine receptors (Jacobson and Gao, 2006).

Adenosine $A_{2A}R$ receptor is a G-protein coupled receptor and it mediates their activity via Gs-protein in peripheral systems or Golf protein in the striatum. Activation of A_{2A} receptors increases adenylyl cyclase activity. It has been reported that adenosine $A_{2A}R$ is involved in vasodilation in the aorta and coronary artery (Fredholm et al., 2001). Moreover, activation of $A_{2A}R$ results in tachycardic effects by centrally located receptors and hypotensive effects by peripheral receptors (Schindler et al., 2005). These findings increase the efforts for treating hypertension with adenosine $A_{2A}R$ agonist CGS 21680; however, due to its non-target effects, the clinical applications of it terminated in vivo studies.

The effects of caffeine, a general adenosine receptors antagonist, in the central nervous system such as enhancement of awareness and learning lead to researchers to investigate selective adenosine receptors antagonists in the nervous system. These investigations revealed that adenosine A_1R and $A_{2A}R$ involves in the effects of caffeine in the central nervous system (Solinas et al., 2005; Ledent et al., 1997a). Especially, deletion of $A_{2A}R$ by genetic engineering techniques elucidate caffeine mediates its behavioral stimulatory effects by adenosine $A_{2A}R$ and has a crucial role in sleep regulation (Fredholm et al., 2004; Huang et al., 2005; Satoh et al., 1998). Besides that, many reports indicate that lack of the dopamine release in the striatum caused some Parkinson's diseases symptoms. Remarkably, evidence shows that the interaction between dopamine D_2R and adenosine

$A_{2A}R$ is antagonistic in the striatum. Striatum includes the neurons that co-expressed $A_{2A}R$ and D_2R and heterodimerization of these two subtypes restrains D_2R function (Fredholm et al., 2001; Ferre et al., 1991). Therefore, $A_{2A}R$ antagonist application thought to be an alternative solution for treatment of the Parkinson's disease. Later clinical studies revealed that KW-6002 (istradefylline), an $A_{2A}R$ antagonist, is a novel treatment for the Parkinson's disease (Xu et al., 2005; Hauser et al., 2003; Weiss et al., 2003). In addition, studies showed that $A_{2A}R$ agonist could play neuroprotection roles in some experimental conditions and cerebral hemorrhagic injury (Mayne et al., 2001).

The reports demonstrate that ethanol increases brain levels of adenosine by inhibiting adenosine reuptake. Then, alcohol and adenosine interact synergistically with the activation of D_2 receptors in median spiny neurons of the striatum/nucleus accumbens, unlike the antagonistic relationship between dopamine and adenosine. Since G-protein β and γ dimers inhibition reduces voluntary alcohol consumption, antagonize the synergism of the $A_{2A}R$ and D_2R with a drug might be a solution for alcohol control (Nature review-Kenneth). Also, a selective $A_{2A}R$ antagonist can show antidepressant effects (El Yacoubi et al., 2001).

In addition, $A_{2A}R$ activation by an agonist can also show protection roles in the peripheral system such as kidney (Linden, 2005). Especially, ischemic injury in the kidney prevents by the $A_{2A}R$ in bone marrow-derived cells (Fredholm et al., 2001). Moreover, studies showed that activation of $A_{2A}R$ by selective agonist CGS 21680 provide board spectrum anti-inflammatory activity in a model of allergic asthma in the

rat. These findings suggesting that $A_{2A}R$ agonist could be useful alternatives to glucocorticosteroids in the treatment of asthma (Fozard et al., 2002). However, later dose studies of GW328267, an $A_{2A}R$ agonist designed for intranasal administration was negative. Therefore, compound withdrew from the clinical testing (Selective A2a receptor agonists as inhibitors of cellular activation, 2000)

Activation of $A_{2A}R$ via adenosine is almost found all immune cells, including lymphocytes, monocytes, macrophages, and dendritic cells (Sitkovsky et al., 2004) and it seems to attenuate inflammation and reperfusion injury in a selection of tissues. Reports show that $A_{2A}R$ importantly involved in the limitation and termination of the prolonged inflammation (Ohta and Sitkovsky, 2001).

I.7 Objective and outline of the dissertation

Insomnia is one of the most common sleep problems with an estimated prevalence of 10–15% in the general population and 30–60% in the older population (Roth, 2007). The most widely prescribed agents for the treatment of insomnia are central nervous system depressants, known as benzodiazepines (BDZ) and non-BDZ, that enhance signaling of the inhibitory neurotransmitter γ-aminobutyric acid (Wafford and Ebert, 2008). These medications are, however, plagued by a wide range of adverse effects, including muscle relaxation, rebound insomnia, changes in appetite, next-day sedation, cognitive impairment, amnesic effects, and the development of drug tolerance and dependence (Aragona, 2000; Vgontzas et al., 1995). In addition, orexin receptor antagonists have most recently been developed and approved as a medication for insomnia treatment (Cox

et al., 2010). The major issues of these drugs are, however, next-morning sleepiness with possible signs of muscle weakness, weird dreams, sleepwalking, other nighttime behaviors or suicidal ideation (Jacobson et al., 2014). Moreover, because orexin receptor antagonists mostly work by preventing arousal from sleep, they are widely inefficient in people who have problems falling asleep.

CGS 21680, a highly selective adenosine A_{2A} receptor ($A_{2A}R$) agonist, produces profound increases in sleep after infusion into the subarachnoid space underlying the ventral surface region of the rostral basal forebrain in rats or into the lateral ventricle of mice (Satoh et al., 1999; Scammell et al., 2001; Urade et al., 2003). However, it is commonly believed that administration of an $A_{2A}R$ agonist has no clinical potential for treating sleep disorders because of its cardiovascular effects, including hypotension and tachycardia (de Lera Ruiz et al., 2014). Selective physiologic $A_{2A}R$ responses may, however, be evoked by a positive allosteric modulator, because its action, in contrast to an agonist, is limited to when and where adenosine is released. Adenosine levels in the brain are elevated during sleep (Porkka-Heiskanen et al., 1997) and thus, allosteric modulation may be an alternative strategy for the treatment of insomnia. In this study, we identified a positive allosteric modulator for $A_{2A}R$, termed $A_{2A}R$ PAM-1, induces slow-wave sleep (SWS) in mice without affecting body temperature and cardiovascular function.

Chapter 1

An identification of adenosine $A_{2A}R$ positive allosteric modulator by small-molecule compounds screening

1.1 Introduction

Adenosine A_{2A} receptors are the member of the G-protein coupled receptors family and coupling with Gs protein. Activation of A_{2A}R induces synthesis of the secondary messenger; cyclic amino monophosphate (cAMP). We established mouse A_{2A}R expressing Chinese hamster ovary cells (CHO) by using retroviral methods for screening small molecules from Nagase laboratory compounds library at the International Institute for Integrative Sleep Medicine, University of Tsukuba. High throughput screening revealed an adenosine A_{2A}R positive allosteric modulator. Then, series of experiments were conducted to investigate allosteric characteristic of this modulator.

1.2 Materials and Methods

1.2.1 Mouse A_{2A}R-expressing CHO cells

The flag epitope tagged open reading frame of A_{2A}R was amplified by PCR from mouse brain total RNA. The resultant amplicon was cloned into a pMXs-IRES-Puro retroviral vector (Kitamura et al., 2003). Subsequently, this plasmid was transfected into the retrovirus packaging cell line Plat-E (Morita et al., 2000). The supernatant of transfected Plat-E cells was recovered after 24h and applied to Chinese hamster ovary (CHO) cells strongly expressing the ecotropic receptor for the retrovirus (Montminy et al., 1990). Mouse A_{2A}R-expressing CHO (mA_{2A}R-CHO) cells were selected in Dulbecco's modified eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 1% nonessential amino acids (NEAA) by treatment with hygromycin B (250 μg/ml) and puromycin (10 μg/ml). The mA_{2A}R-CHO cells were subsequently maintained in DMEM

supplemented with 5% FBS, 1% NEAA, 1% penicillin/streptomycin, 250 µg/ml Hygromycin B at 37 °C in an atmosphere of 5% CO_2.

1.2.2 cAMP assay

Activation of $A_{2A}R$ was quantified by cyclic-adenosine monophosphate (cAMP) accumulation in CHO cells expressing mouse $A_{2A}R$. CHO cells were suspended in Hanks' balanced salt solution (HBSS) containing 1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.25 M IBMX (3-isobutyl-1-methylxanthine) in 384-well micro-plates (2×10^3 cells per well) and incubated with adenosine, CGS 21680, $A_{2A}R$ PAM-1 at indicated concentrations for 30 min at 25^{o}C. The detection mixture containing Eu-cAMP tracer and ULight-anti-cAMP antibody was added and incubated for 1h at 25^{o}C. Micro-plate reader (ARVOX5; excitation: 340 nm; emission: 665 nm) was used to measure Forster resonance energy transfer (FRET) signal. All experiments were done under the guidelines of the manufacturer's instruction. (LANCE Ultra cAMP Kit, PerkinElmer). The cAMP levels are based on the dynamic range ("linear portion") of the cAMP standard curve and normalised to the baseline or adenosine treated group.

1.2.3 Synthesis of $A_{2A}R$ PAM-1

A solution of 2,3,4-fluorobenzoic acid (1.35 g, 7.68 mmol), 2-fluoro-4-iodoaniline (1.91 g, 8.06 mmol), and lithium amide (0.702 g, 30.6 mmol) in tetrahydrofuran (10.5 mL) was reacted using a standard method (Cai et al., 2008) to give 3,4-difluoro-2-((2-fluoro-4-iodophenyl)amino)benzoic acid [$A_{2A}R$ positive allosteric modulator (PAM)-1, 2.99 g, 99%] as a brown solid (**Figure 1.6)**; IR (KBr) 3311, 1673, 1602, 1520, 1500, 1444, 1273, 768 cm^{-1}; ^{1}H NMR (400 MHz CD_3OD) δ = 7.89 (1 H, ddd, *J* = 2.3, 6.0, 9.2 Hz), 7.48 (1

H, dd, J = 1.8, 10.5 Hz), 7.41 (1 H, ddd, J =1.4, 1.8, 8.5 Hz), 6.91 (1 H, ddd, J = 7.3, 9.4, 9.4 Hz), 6.75 (1 H, ddd, J = 5.6, 8.5, 8.5 Hz); ^{13}C NMR (100 MHz acetone-d_6) δ = 169.9, 155.7 (dd, $J_{C,F}$ = 252.1, 4.8 Hz), 155.6 (d, $J_{C,F}$ = 252.1 Hz), 143.6 (dd, $J_{C,F}$ = 247.8, 14.9 Hz), 137.4 (dd, $J_{C,F}$ = 7.7, 2.9 Hz), 135.0 (d, $J_{C,F}$ = 3.8 Hz), 131.9(d, $J_{C,F}$ = 11.5 Hz), 129.8 (dd, $J_{C,F}$ = 9.6, 3.8 Hz), 125.8 (d, $J_{C,F}$ = 21.0 Hz), 123.8 (d, $J_{C,F}$ = 5.8 Hz), 116.4, 110.1 (d, $J_{C,F}$ = 18.2 Hz), 84.7 (d, $J_{C,F}$ = 6.7 Hz); HRMS-ESI: *m/z* [M-H]$^-$ calcd for C13H6F3INO2, 391.9395; measured, 391.9414.

1.3 Results

1.3.1 Establishment of mA$_{2A}$R expressing Chinese hamster Ovary (CHO) cells

We established CHO cells that express mouse A$_{2A}$R by a retrovirus mediated gene transfer method (**Figure 1.1**) (Kitamura et al., 2003). We then decided to use two thousands mA$_{2A}$R-CHO cells per well for further compounds screening test after checking the baseline cAMP level of the different cell numbers by using the förster resonance energy transfer (FRET) immunoassay (**Figure 1.1A**). In addition, we observed increased level of the cAMP in mA$_{2A}$R-CHO cells after treatment with different adenosine concentrations and also mA$_{2A}$R expression in CHO cells were checked by quantitative polymerase chain reactions (QPCR) (**Figure 1.1B, C**). These experiments revealed that mA$_{2A}$R highly expressed in CHO cells and they significantly responded to adenosine treatment.

1.3.2 Screening of small-molecule compounds for allosteric $A_{2A}R$ modulation

Subsequently, we used these $mA_{2A}R$-CHO cells to screen 1173 small-molecule compounds for their allosteric effects at $A_{2A}Rs$. The compounds were synthesized in Dr. Hiroshi Nagase's laboratory at the University of Tsukuba. $A_{2A}R$ activity in CHO cells was determined by measuring cyclic adenosine monophosphate (cAMP) level after the 30 min incubation with adenosine and small-molecule compounds using förster resonance energy transfer immunoassay. Initially, we tested 391 mixtures that had 3 compounds each in a primary screening. The results show that eight mixtures significantly enhanced the adenosine effects at $A_{2A}R$ (**Figure 1.2**).

1.3.3 Positive allosteric effects of $A_{2A}R$ PAM-1 on $mA_{2A}R$-CHO cells

Further individual testing of the compounds 370, 371, and 372 in mixture 124 revealed that only compound 371 (3,4-difluoro-2-((2-fluoro-4-iodophenyl)amino)benzoic acid) in mixture 124 enhanced adenosine-induced $A_{2A}R$ activation (**Figure 1.3A**). A cell culture bioassay revealed that cAMP levels were not altered by treating $A_{2A}R$-expressing or native CHO cells with compound 371 in the absence of adenosine or by treating native CHO with adenosine and compound 371 (**Figure 1.3B**), suggesting that compound 371 is likely a positive allosteric modulator for $A_{2A}R$ and thus, we named this compound $A_{2A}R$ PAM-1 (**Figure 1.3A**).

1.3.4 Allosteric modulation of $A_{2A}R$ by $A_{2A}R$ PAM-1

Co-treatment of $A_{2A}R$-expressing CHO cells with 150 µM adenosine and different concentrations of $A_{2A}R$ PAM-1 (i.e., 25, 50, and 100 µM) amplified adenosine $A_{2A}R$-evoked cAMP accumulation in a dose-dependent manner by 42 % ± 1.4, 46 % ± 1.1, and 50 % ± 1.0, respectively (**Figure 1.4A**). Similarly, co-treatment of $A_{2A}R$-expressing CHO cells with 100 µM $A_{2A}R$ PAM-1 and 50, 100, or 150 nM adenosine increased $A_{2A}R$ activity in the CHO cells in a dose-dependent manner by 55 % ± 0.4, 66 % ± 1.5, and 72 % ± 1.7, whereas 100 µM $A_{2A}R$ PAM-1 did not significantly enhance the cellular activity of $A_{2A}R$-expressing CHO cells treated with 250 nM adenosine (**Figure 1.4B**). Surprisingly, co-treatment of m$A_{2A}R$-CHO cells with 100 µM $A_{2A}R$ PAM-1 and different concentrations of the highly selective $A_{2A}R$ agonist CGS 21680 did not increase the level of the cAMP (**Figure 1.5**).

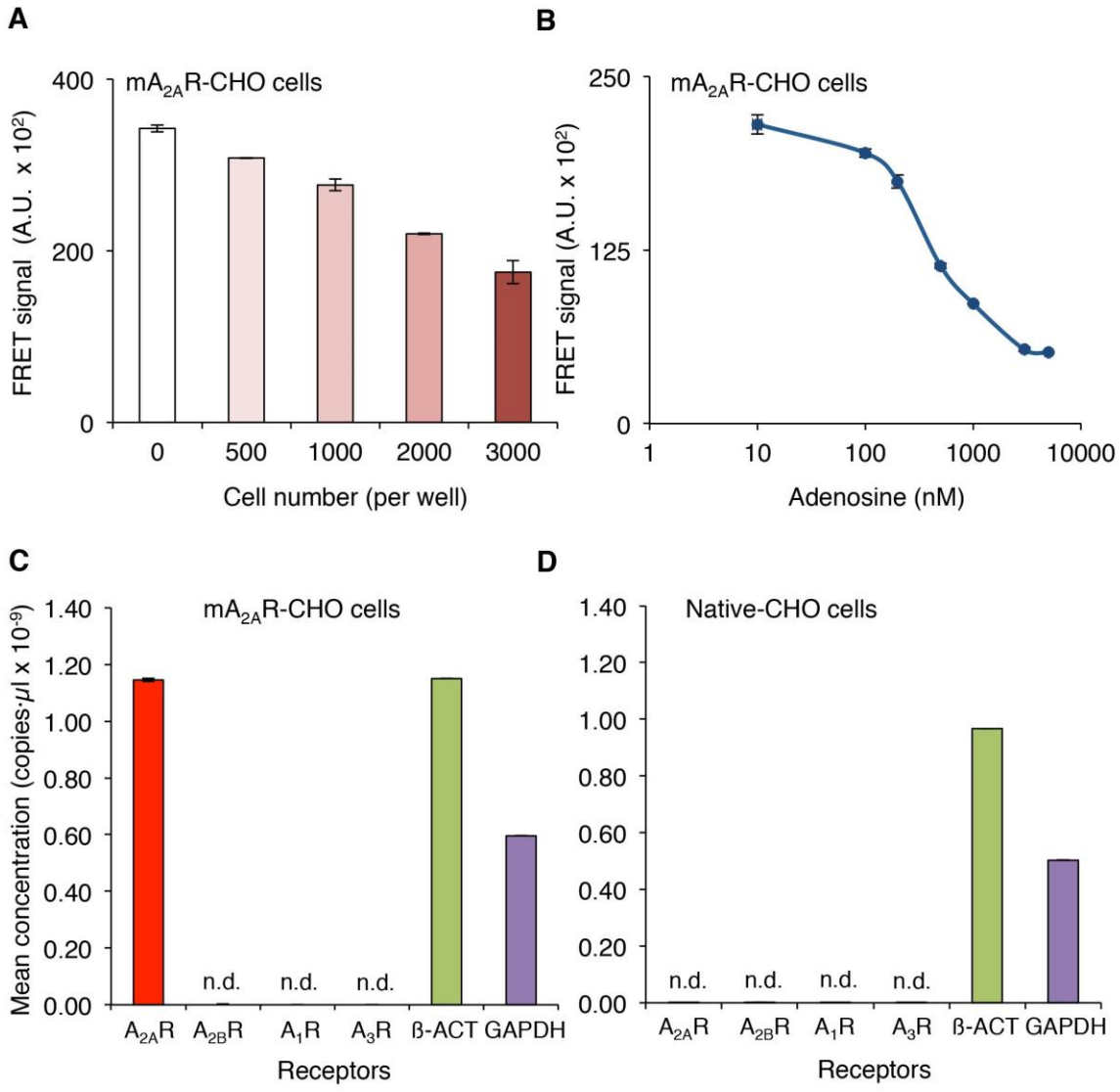

Figure 1.1 Characterization of mouse A_{2A}R-expressing CHO cells.

(A) Cell number-dependent FRET activity of mA_{2A}R-expressing CHO. **(B)** Dose-dependent changes of FRET activity in mA_{2A}R-expressing CHO after adenosine administration. **(C)** Expression of Chinese hamster adenosine receptors in mA_{2A}R-expressing (left panel) and native (right panel) CHO cells.

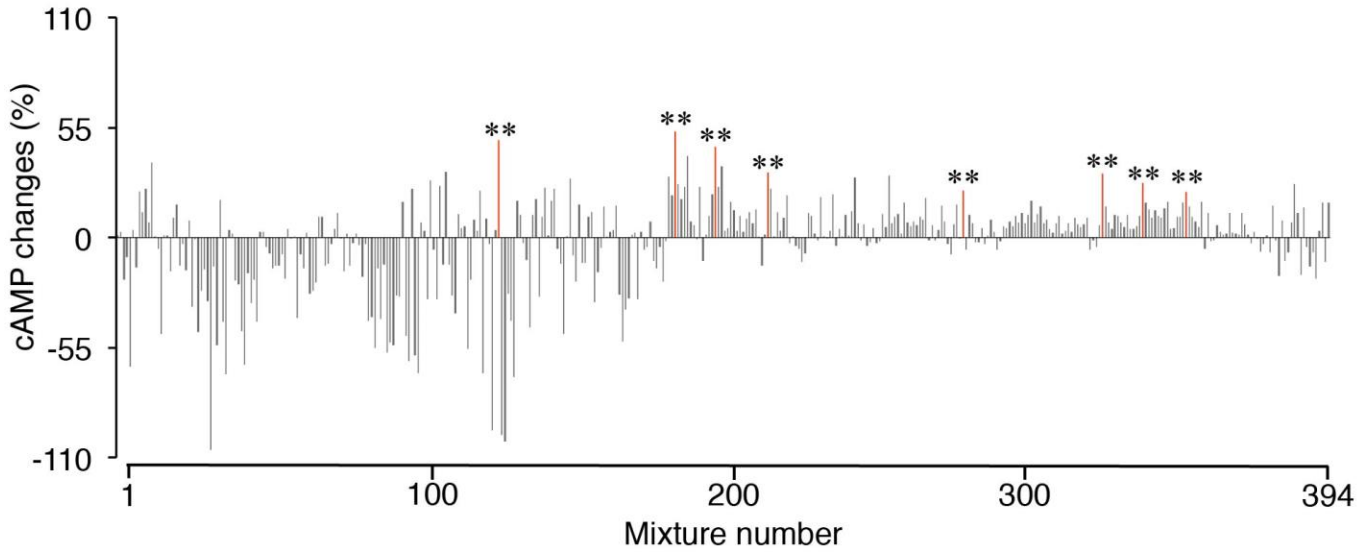

Figure 1.2 High-throughput screening of small-molecule compounds

Changes of cAMP levels in CHO cells after treatment with adenosine and compound mixtures are shown as percentage of cAMP levels in CHO cells after treatment with adenosine. Experiments were performed in triplicate wells for each condition and repeated at least twice. $^{**}P < 0.01$, compared with adenosine, assessed by unpaired two-tailed Student's *t*-test.

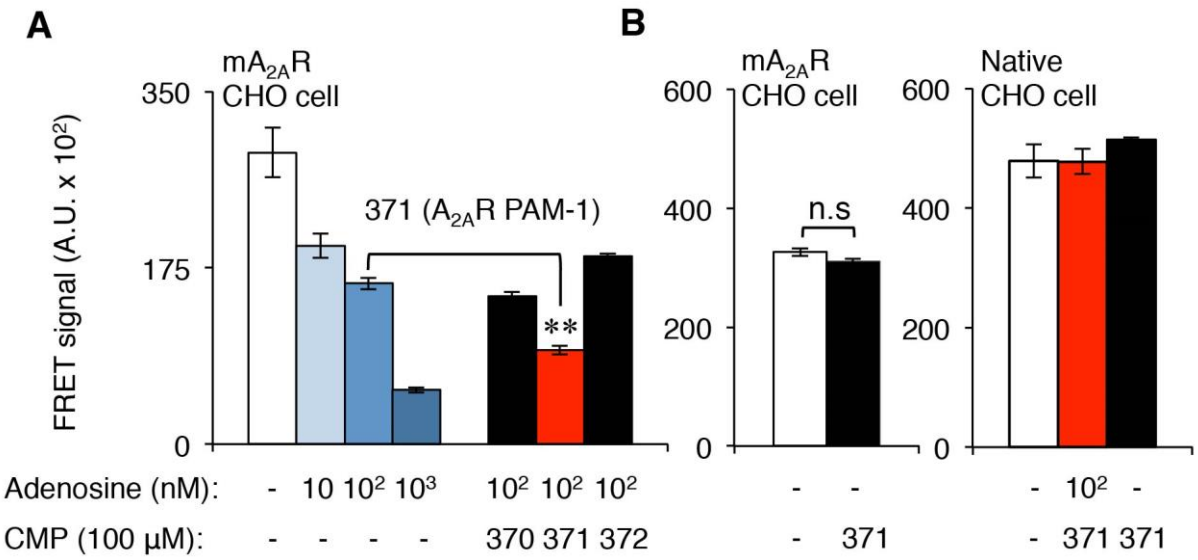

Figure 1.3 Co-treatment of $mA_{2A}R$-CHO cells with $A_{2A}R$ PAM-1 and adenosine revealed an allosteric modulation

(A) FRET activity in $mA_{2A}R$-expressing CHO after treatment with adenosine and small molecule compounds 370, 371, or 372. (**B**) FRET activity in $mA_{2A}R$-expressing (left panel) and native (right panel) CHO cells after treatment with adenosine or adenosine and $A_{2A}R$ PAM-1, respectively. Experiments were performed in triplicate wells for each condition and repeated at least twice. Data are presented as the mean ± SEM. **: $p<0.01$ compared with adenosine, assessed by unpaired Student's *t*-test.

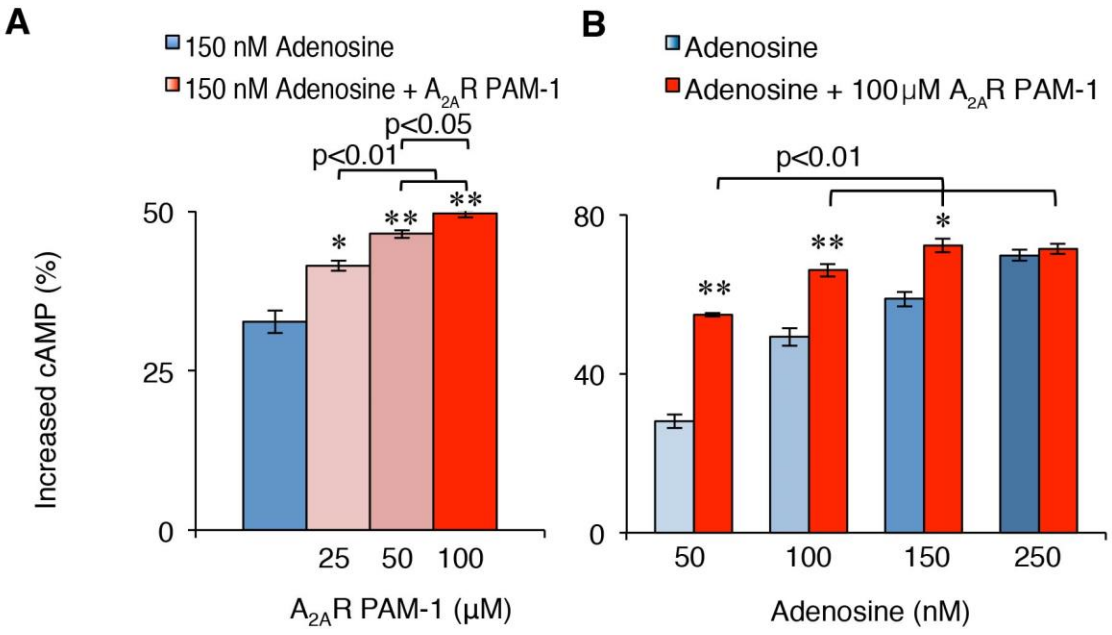

Figure 1.4 Allosteric modulation of $A_{2A}R$ by $A_{2A}R$ PAM-1

Dose-dependent changes of cAMP level in $mA_{2A}R$-expressing CHO cells after treatment with adenosine and different concentrations of $A_{2A}R$ PAM-1 (**A**) or $A_{2A}R$ PAM-1 and different concentrations of adenosine (**B**). Experiments were performed in triplicate wells for each condition and repeated at least twice. Data are presented as the mean ± SEM. *: $p<0.05$, **: $p<0.01$ compared with adenosine, assessed by unpaired Student's *t*-test.

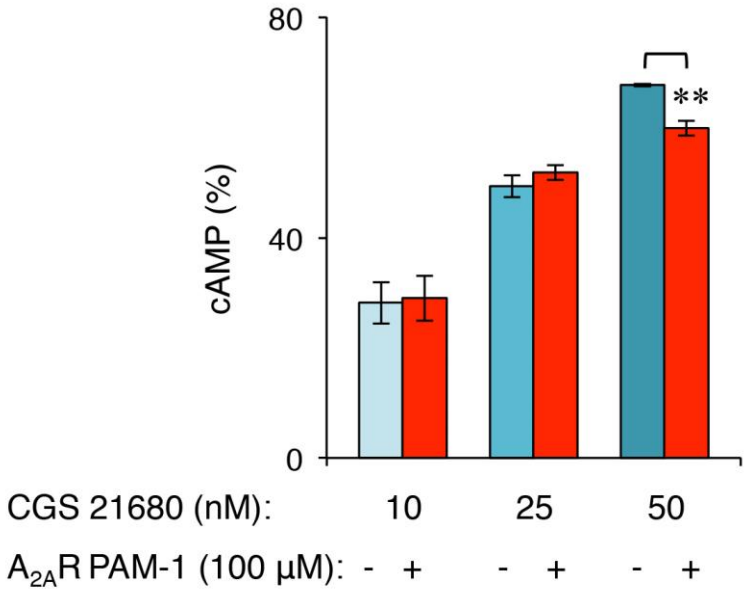

Figure 1.5 $A_{2A}R$ PAM-1 does not enhance $A_{2A}R$ activity in the presence of $A_{2A}R$ agonist CGS 21680.

Changes of cAMP levels in $mA_{2A}R$-expressing CHO cells after co-treatment with $A_{2A}R$ PAM-1 and different concentrations of $A_{2A}R$ agonist CGS 21680. Data are presented as the mean ± SEM. **: $p<0.01$ compared with CGS 21680 alone, assessed by unpaired Student's *t*-test.

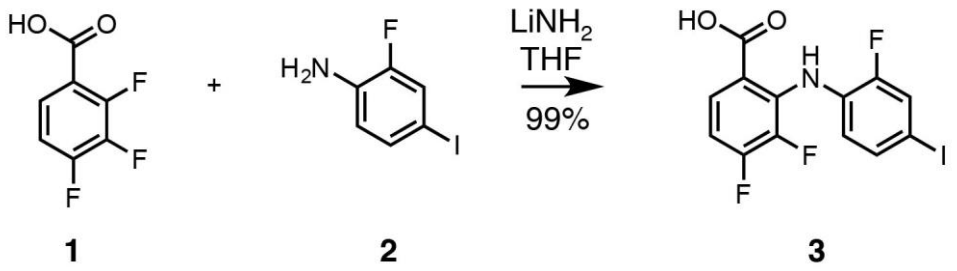

Figure 1.6 Chemical synthesis of $A_{2A}R$ PAM-1.

$A_{2A}R$ PAM-1 (3) was produced by combining 2,3,4-fluorobenzoic acid (1) and 2-fluoro-4-iodoaniline (2).

1.4 Summary & Conclusions

Small molecules screening by using mouse $A_{2A}R$ ($mA_{2A}R$) expressing CHO cells revealed a positive allosteric modulator for adenosine $A_{2A}R$, denoted $A_{2A}R$ PAM-1. Further cell culture experiments showed that $A_{2A}R$ PAM-1 dose-dependently enhanced the activity of adenosine at $A_{2A}R$. The enhancement activity of $A_{2A}R$ PAM-1 did not observe the high concentration of adenosine treatment (i.e. 250 nM). In addition, $A_{2A}R$ PAM-1 did not enhance the activity of $A_{2A}R$ potent agonist CGS 21680. This result indicates that CGS 21680 may block the binding site of $A_{2A}R$ PAM-1 at A_{2A} receptor.

Chapter 2

Intraperitoneal administration of $A_{2A}R$ PAM-1 effects on slow-wave sleep in mice

2.1 Introduction

Since cell culture experiments revealed an adenosine $A_{2A}R$ positive allosteric modulator ($A_{2A}R$ PAM-1) and it has known that central stimulation of $A_{2A}R$ induces slow-wave sleep, we then decided to test sleep-inducing effects of the $A_{2A}R$ PAM-1 in mice. Sleep bioassay was used to analyze wake, SWS, and REM sleep periods of the mice after intraperitoneal administration of adenosine $A_{2A}R$ allosteric modulator. In addition, we conducted a dose dependency test for $A_{2A}R$ PAM-1 and analyzed the sleep architecture after the i.p. administration of $A_{2A}R$ PAM-1 in mice.

2.2 Materials and Methods

2.2.1 Animals

Male mouse lines on a C57BL/6 background, including wild-type and $A_{2A}R$ KO (Chen et al., 1999) mice, which were maintained at the International Institute of Integrative Sleep Medicine and weighing 21-27 g (10-15 weeks old), were used in the experiments. The animals were housed in an insulated and soundproof recording chamber that was maintained at an ambient temperature of 23 ± 0.5°C with a relative humidity of 50 ± 5% and an automatically controlled 12 h light/12 h dark cycle (light on at 8:00, illumination intensity ≈ 100 lux). All animals had free access to food and water. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (2011). Experimental protocols were in compliance with relevant Japanese and institutional laws and guidelines and approved by the University of Tsukuba animal ethics committee (protocol #14-322).

Every effort was made to minimize the number of animals used as well as any pain and discomfort experienced by the animals

2.2.2 Stereotaxic surgery for placement of EEG/EMG electrodes

Mice were anesthetized with pentobarbital [50 $mg \cdot kg^{-1}$, intraperitoneal (i.p.)] and then placed in a stereotaxic apparatus. Electroencephalogram (EEG) and electromyogram (EMG) electrodes for polysomnographic recordings were chronically implanted in the mice (Oishi et al., 2016). The implant comprised two stainless steel screws (1 mm in diameter) inserted through the skull above the cortex (anteroposterior, +1.0 mm; left-right, -1.5 mm from bregma or lambda) according to the atlas of Paxinos and Franklin (Paxinos and Franklin, 2004) that served as the EEG electrodes. Two insulated, stainless steel Teflon-coated wires were placed bilaterally into both trapezius muscles and served as the EMG electrodes. All electrodes were attached to a micro connector and fixed to the skull with dental cement.

2.2.3 Vigilance state assessment based on EEG/EMG polygraphic recordings

Ten days after surgery, the mice were individually housed in transparent barrels in an insulated soundproof recording chamber and connected to the EEG-EMG recording cables for 3 to 5 days of habituation before starting the polygraphic recordings. To evaluate the spontaneous sleep-wake cycle, each animal was recorded for 24 h beginning at 20:00, the onset of the dark period. The animals then entered the pharmacologic phase of the study in which sleep-wakefulness parameters were recorded for 36 h. The data collected during the first 24 h also served as baseline comparison data for the second experimental day. Cortical EEG/EMG recordings were amplified, filtered (EEG 0.5-30

Hz; EMG 20-200 Hz), and digitized at a sampling rate of 128 Hz, and then recording using data acquisition software SleepSign® (Kissei Comtec, Matsumoto, Japan). The vigilance states were classified offline in 10-s epochs into three stages, i.e., wakefulness, rapid eye movement (REM) sleep, and SWS by SleepSign® (ver 3.4) according to standard criteria (Oishi et al., 2016). As a final step, defined vigilance stages were examined visually, and corrected when necessary.

2.2.4 Pharmacological treatment

For control data, mice were injected with saline or vehicle (10 ml·kg^{-1} body weight, i.p.) at 22:00 or 21:30, respectively. A_{2A}R PAM-1 was dissolved in saline immediately before use and administered intraperitoneally at 22:00 on the experimental day at a dose of 30, 60, or 75 mg·kg^{-1}. Mice were randomly assigned to groups that received control or drug injections.

2.2.5 Formation of the sodium salt of A_{2A}R PAM-1

Aqueous sodium hydroxide (100 μM, 754 μL) was added to a stirred solution of A_{2A}R PAM-1 (0.266 g, 75.4 mmol) in ethanol (20.0 mL) at 0°C. The mixture was stirred for 45 min at room temperature and then concentrated in vacuo and freeze-dried. The residue was dissolved in water and filtered. The filtrate was freeze-dried to obtain the sodium salt of A_{2A}R PAM-1 (0.265 g, 89%) as a gray solid (m.p. 290−291°C; Anal. Calcd for $C_{13}H_6NO_2 \cdot Na \cdot 1.5H_2O$: C, 35.32; H, 2.05; N, 3.17. Measured: C, 35.34; H, 1.91; N, 3.14). The sodium salt of A_{2A}R PAM-1 was used for all *in-vivo* experiments.

2.3 Results

2.3.1 Intraperitoneal administration of $A_{2A}R$ PAM-1 induces SWS in mice

We then tested the effect of intraperitoneal administration of $A_{2A}R$ PAM-1 on the sleep/wake behavior of wild-type mice. We analyzed EEG and EMG recordings made after saline or $A_{2A}R$ PAM-1 injections during the dark period at 22:00, when mice usually spend most of their time awake. Whereas baseline sleep and wake of mice prior treatment was not significantly different between the saline and $A_{2A}R$ PAM-1 groups during the dark period (**Figure S3**), $A_{2A}R$ PAM-1 (75 $mg \cdot kg^{-1}$) increased SWS after the injections for the following 8 h (**Figure 2.1A,B; Figure 2.2A**)

The total amount of SWS was increased by 60.8 ± 11.4 min for 8 h with the highest dose of $A_{2A}R$ PAM-1 (i.e., 75 $mg \cdot kg^{-1}$) compared with saline treatment, whereas wakefulness was decreased by 59.2 ± 12.8 min (**Figure 2.2B**). Intraperitoneal injection of $A_{2A}R$ PAM-1 did not significantly alter the REM sleep duration during the dark period compared with saline injection.

2.3.2 Intraperitoneal administration of $A_{2A}R$ PAM-1 induces SWS in a dose dependent manner

The $A_{2A}R$ PAM-1 dose-dependently increased SWS, which is also known as non-rapid eye movement (non-REM) sleep, the major part of sleep characterized by slow and high-voltage brain waves, for up to 8 h (**Figure 2.3A**). To assess whether EEG activity was altered by $A_{2A}R$ PAM-1 administration, we compared the normalized EEG power spectrum of SWS in mice treated with saline or $A_{2A}R$ PAM-1 (**Figure 2.3B**). EEG

activity in the frequency range of 0.5–25 Hz during SWS was indistinguishable between A_{2A}R PAM-1–induced and natural (saline injection) SWS. These data suggest that A_{2A}R PAM-1 induced physiologic sleep rather than abnormal sleep.

2.3.3 Intraperitoneal administration of A_{2A}R PAM-1 effects on sleep architecture of mice

Administration of A_{2A}R PAM-1 (75 mg·kg^{-1}, i.p.) to the mice did not significantly affect the episode numbers of SWS and REM sleep for 8 h in the dark period (**Figure 2.4A**). On the other hand, wake episode numbers lasting 120 to 239 s increased by 307% , wake episode numbers lasting 480 to 959 s and 960 to 1909 s decreased by 47% and 88%, respectively, compared with the saline injection. The mean duration of wake episodes decreased by 38% compared with saline, but the duration of the SWS and REM sleep episodes was not significantly different after A_{2A}R PAM-1 (75 mg·kg^{-1}, i.p.) administration (**Figure 2.4B**). A_{2A}R PAM-1 (75 mg·kg^{-1}, i.p.) also did not significantly affect the number of transitions between SWS, wake, and REM sleep (**Figure 2.4C**).

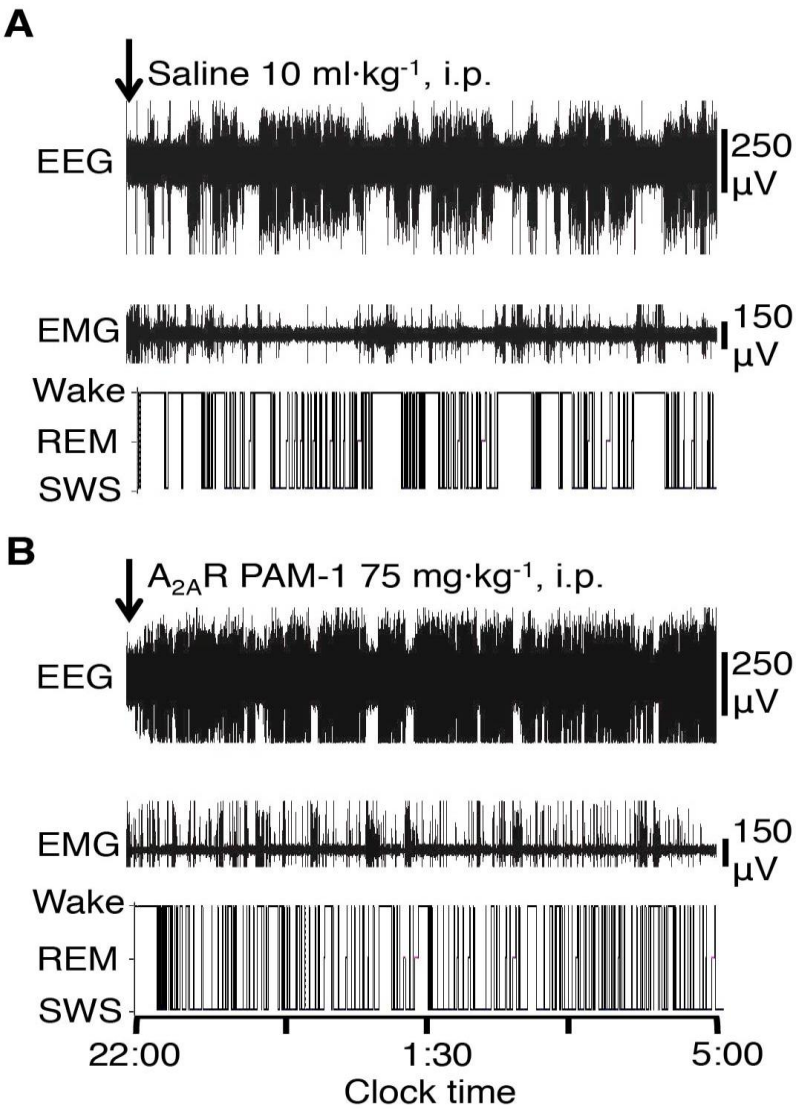

Figure 2.1 Intraperitoneal administration of A_{2A}R PAM-1 induced SWS in mice. Typical examples of EEG, EMG and hypnograms of a mouse after the administration of saline (**A**) or A_{2A}R PAM-1 (**B**). EEG signal of brain shows high amplitude and low frequency in SWS or low amplitude and high frequency in wake periods.

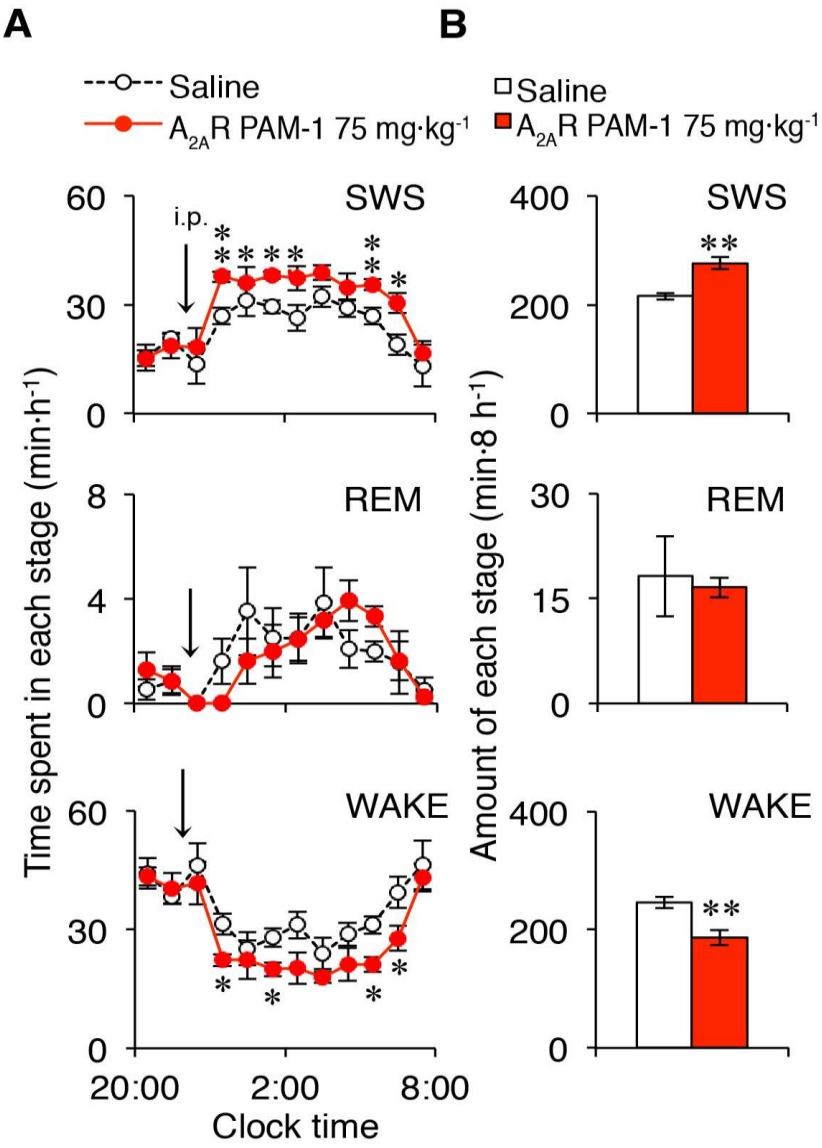

Figure 2.2 Intraperitoneal administration of A_{2A}R PAM-1 increased the time spent and total amount of SWS, REM, and Wake in mice

Time-courses (**A**) and total amounts (**B**) of SWS, REM sleep, and wakefulness in mice after intraperitoneal administration of saline or A_{2A}R PAM-1. Data are presented as the mean ± SEM. *: $p<0.05$; **: $p<0.01$ compared with saline, assessed by ANOVA Tukey Test or unpaired Student's *t*-test, (n=5/group)

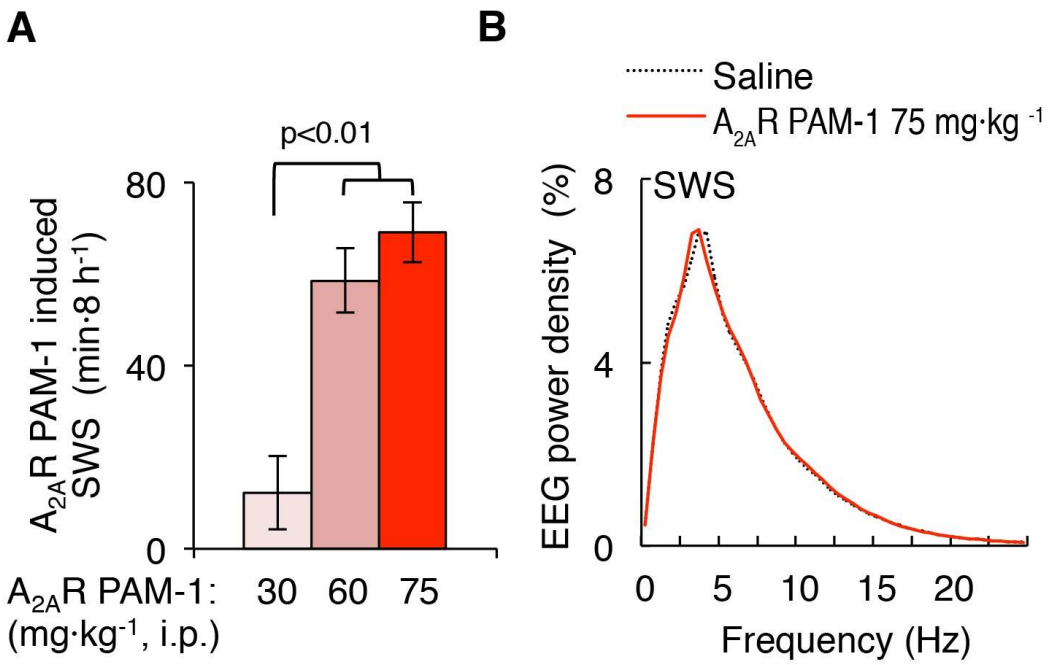

Figure 2.3 Intraperitoneal administration of A_{2A}R PAM-1 induced SWS in a dose-dependent manner with an identical EEG power density as natural sleep.

Dose-dependent changes in SWS time during 8 h after A_{2A}R PAM-1 administration **(A)**. EEG power density of SWS during 8 h after saline or A_{2A}R PAM-1 administration **(B)**. Data are presented as the mean ± SEM and assessed by ANOVA Tukey Test or unpaired Student's *t*-test, (n=5/group)

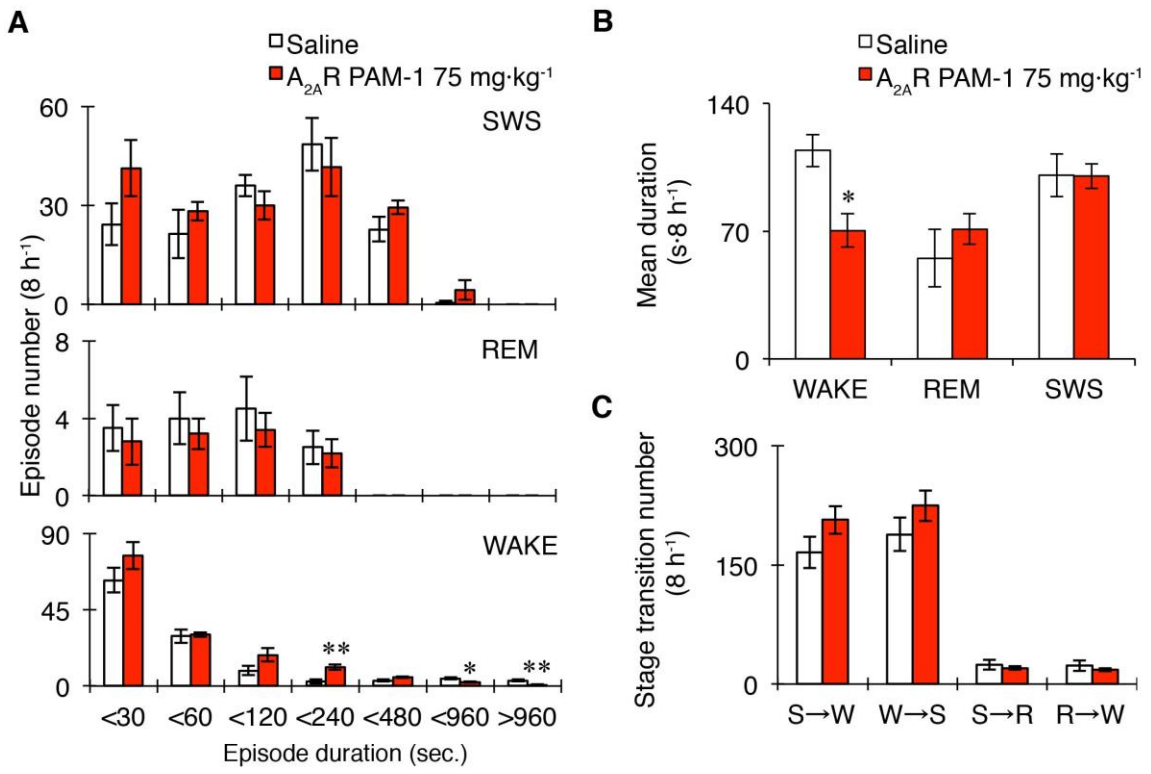

Figure 2.4 Sleep architecture of mice after intraperitoneal administration of $A_{2A}R$ PAM-1.

Episode number **(A)** and mean duration **(B)** of each stage after administration of saline or $A_{2A}R$ PAM-1. (**C**) Transitions between SWS (S), REM sleep (R) and wake (W) stages after administration of saline or $A_{2A}R$ PAM-1. Data are presented as the mean ± SEM. *: $p<0.05$; **: $p<0.01$, compared with saline, assessed by unpaired Student's *t*-test, (n=5/group).

2.4 Summary & Conclusions

Intraperitoneal administration of adenosine A_{2A} receptors positive allosteric modulator, A_{2A}R PAM-1 induced slow-wave sleep with a concomitant decrease of wakefulness in a dose-dependent manner following 8h. Moreover, EEG power density of SWS after administration of A_{2A}R PAM-1 is identical to the control group. It indicates that A_{2A}R PAM-1 induces a physiological sleep but not an artificial sleep. i.p. administration of A_{2A}R PAM-1 does not affect REM sleep and decreases the mean duration of the wake period without any effects on the mean duration of the SWS and REM sleep.

Chapter 3

Sleep-inducing $A_{2A}R$ PAM-1's dependency on the adenosine $A_{2A}R$ in mice.

3.1 Introduction

Since A_{2A}R PAM-1 induces slow-wave sleep in mice, its dependency on adenosine A_{2A}R needed to clarify. To address this issue, we conducted an experiment in which A_{2A}R antagonist suppressed A_{2A}R PAM-1 SWS inducing effects. Moreover, we also tested the SWS inducing effects of A_{2A}R PAM-1 on A_{2A}R global knockout mice to confirm dependency of A_{2A}R PAM-1 to A_{2A}R in vivo.

3.2 Materials and Methods

3.2.1 Animals

Male mouse lines on a C57BL/6 background, including wild-type and A_{2A}R KO (Chen et al., 1999) mice, which were maintained at the International Institute of Integrative Sleep Medicine and weighing 21-27 g (10-15 weeks old), were used in the experiments. The animals were housed in an insulated and soundproof recording chamber that was maintained at an ambient temperature of 23 ± 0.5°C with a relative humidity of 50 ± 5% and an automatically controlled 12 h light/12 h dark cycle (light on at 8:00, illumination intensity ≈ 100 lux). All animals had free access to food and water. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (2011). Experimental protocols were in compliance with relevant Japanese and institutional laws and guidelines and approved by the University of Tsukuba animal ethics committee (protocol #14-322). Every effort was made to minimize the number of animals used as well as any pain and discomfort experienced by the animals.

3.2.2 Stereotaxic surgery for placement of EEG/EMG electrodes

Mice were anesthetized with pentobarbital [50 $mg \cdot kg^{-1}$, intraperitoneal (i.p.)] and then placed in a stereotaxic apparatus. Electroencephalogram (EEG) and electromyogram (EMG) electrodes for polysomnographic recordings were chronically implanted in the mice (Oishi et al., 2016). The implant comprised two stainless steel screws (1 mm in diameter) inserted through the skull above the cortex (anteroposterior, +1.0 mm; left-right, -1.5 mm from bregma or lambda) according to the atlas of Paxinos and Franklin (Paxinos and Franklin, 2004) that served as the EEG electrodes. Two insulated, stainless steel Teflon-coated wires were placed bilaterally into both trapezius muscles and served as the EMG electrodes. All electrodes were attached to a micro connector and fixed to the skull with dental cement.

3.2.3 Vigilance state assessment by using EEG/EMG polygraphic recordings

Ten days after surgery, the mice were individually housed in transparent barrels in an insulated soundproof recording chamber and connected to the EEG-EMG recording cables for 3 to 5 days of habituation before starting the polygraphic recordings. To evaluate the spontaneous sleep-wake cycle, each animal was recorded for 24 h beginning at 20:00, the onset of the dark period. The animals then entered the pharmacologic phase of the study in which sleep-wakefulness parameters were recorded for 36 h. The data collected during the first 24 h also served as baseline comparison data for the second experimental day. Cortical EEG/EMG recordings were amplified, filtered (EEG 0.5-30 Hz; EMG 20-200 Hz), and digitized at a sampling rate of 128 Hz, and then recording

using data acquisition software SleepSign® (Kissei Comtec, Matsumoto, Japan). The vigilance states were classified offline in 10-s epochs into three stages, i.e., wakefulness, rapid eye movement (REM) sleep, and SWS by SleepSign® (ver 3.4) according to standard criteria (Oishi et al., 2016). As a final step, defined vigilance stages were examined visually, and corrected when necessary.

3.2.4 Pharmacological treatment

For control data, mice were injected with saline or vehicle (10 ml·kg^{-1} body weight, i.p.) at 22:00 or 21:30, respectively. A_{2A}R PAM-1 was dissolved in saline immediately before use and administered intraperitoneally at 22:00 on the experimental day at a dose 75 mg·kg^{-1}. ZM241385 (15 mg·kg^{-1}, i.p.) was dissolved in vehicle (5% DMSO, 5% Cremophor® EL in saline) and injected into C57BL/6J mice at 21:30. Mice were randomly assigned to groups that received control or drug injections.

3.3 Results

3.3.1 Sleep-inducing effect of A_{2A}R PAM-1 was suppressed by blocking A_{2A}Rs

We further investigated whether A_{2A}Rs mediate the sleep-inducing effect of A_{2A}R PAM-1. First, we pretreated wild-type mice with the selective A_{2A}R antagonist ZM241385 (15 mg·kg^{-1}, i.p.) or vehicle 30 min before the A_{2A}R PAM-1 injection at 22:00. The dose of ZM241385 was selected based on previous studies (El Yacoubi et al., 2000; Nakamura et al., 2016). In the presence of ZM241385, A_{2A}R PAM-1 injection produced no significant

changes in SWS (**Figure 3.1A**), indicating that ZM241385 completely blocked the $A_{2A}R$ PAM-1–induced SWS. When we calculated the total amount of SWS for 4 h after the intraperitoneal injection of $A_{2A}R$ PAM-1 (**Figure 3.1B**), we found that it did not significantly alter the total amount of SWS after ZM241385 pretreatment. ZM241385 pretreatment alone also had no significant effect on SWS compared with vehicle pretreatment.

3.3.2 Sleep inducing effects of $A_{2A}R$ PAM-1 was abolished in $A_{2A}R$-KO mice

We then administered 75 $mg \cdot kg^{-1}$ $A_{2A}R$ PAM-1 (i.p.) into $A_{2A}R$ KO mice and their wild-type littermates at 22:00. We observed no significant changes in SWS in the $A_{2A}R$ KO mice compared with saline treatment, whereas SWS was increased by 74.3 ± 12.0 min for 6 h in wild-type littermates of $A_{2A}R$ KO mice (**Figure 3.2A, B**). Concomitantly, wakefulness was decreased in the wild-type littermates of $A_{2A}R$ KO mice, whereas neither REM sleep in these mice nor wakefulness and REM sleep in the KO mice were affected by intraperitoneal administration of 75 $mg \cdot kg^{-1}$ $A_{2A}R$ PAM-1 (**Figure 3.3A-D**). Baseline sleep and wake of knockout mice and their wild-type littermates prior to treatment was not significantly different between the saline and $A_{2A}R$ PAM-1 groups during the dark period (data not shown). These findings suggest that $A_{2A}R$ are necessary for $A_{2A}R$ PAM-1 to induce SWS.

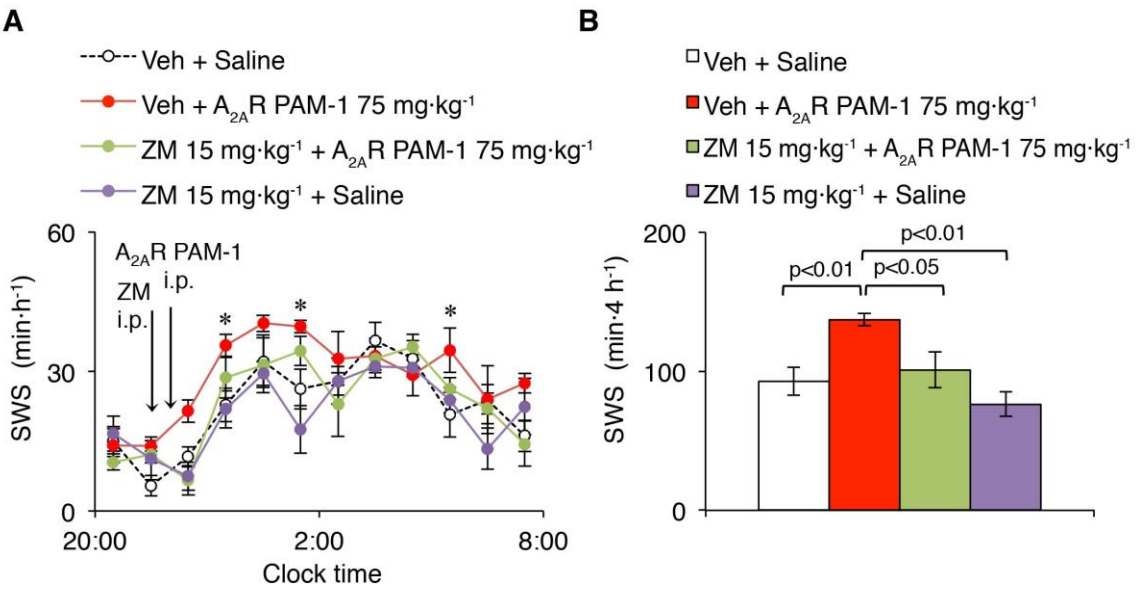

Figure 3.1 A_{2A}R antagonist suppressed sleep-inducing effect of A_{2A}R PAM-1 in mice

Time-courses (**A**) and total amount (**B**) of SWS in mice pretreated with vehicle or A_{2A}R antagonist ZM 241385 after administration of saline or A_{2A}R PAM-1. Data are presented as the mean ± SEM. *: $p<0.05$; compared with saline, assessed by ANOVA Tukey Test or unpaired Student's t-test, (n=5/groups).

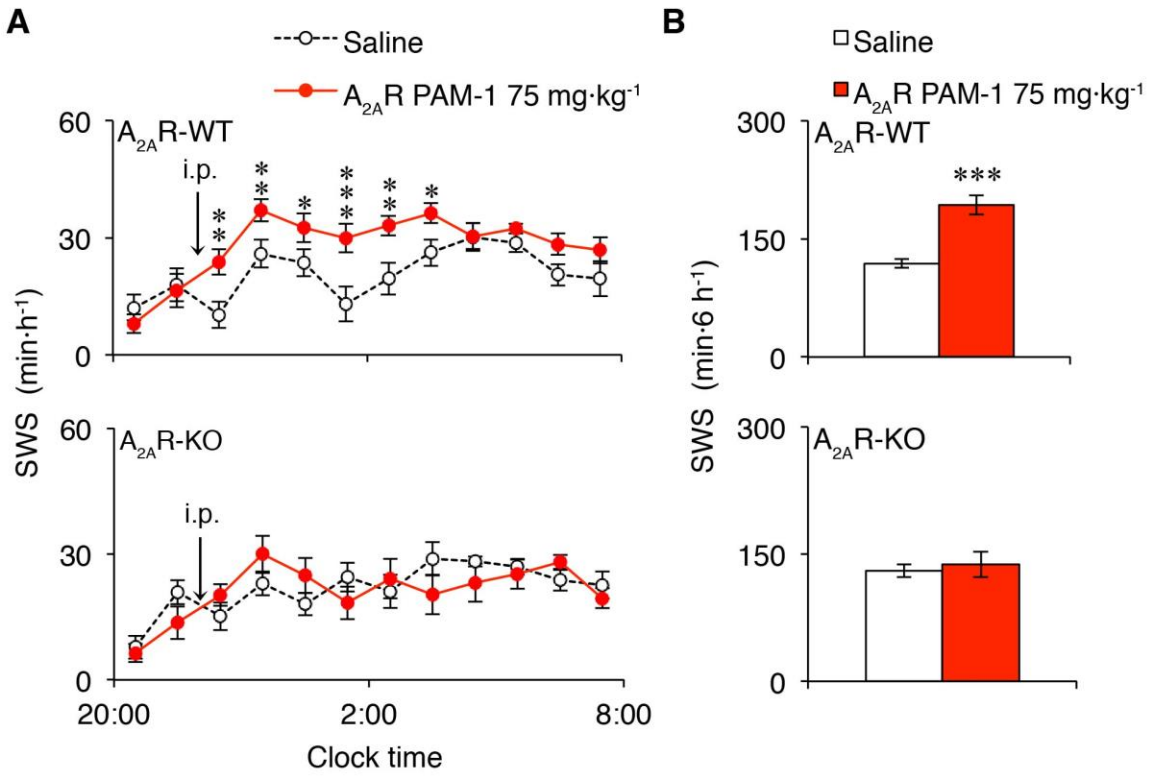

Figure 3.2 Sleep inducing effect of A_{2A}R PAM-1 was abolished in A_{2A}R-KO mice.

Time courses (**A**) and total amount **(B)** of SWS in wild-type (top panels) or A_{2A}R knockout mice (bottom panels) after administration of saline or A_{2A}R PAM-1. Data are presented as the mean ± SEM. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ compared with saline, assessed by ANOVA Tukey Test or unpaired Student's t-test, (n=8/group).

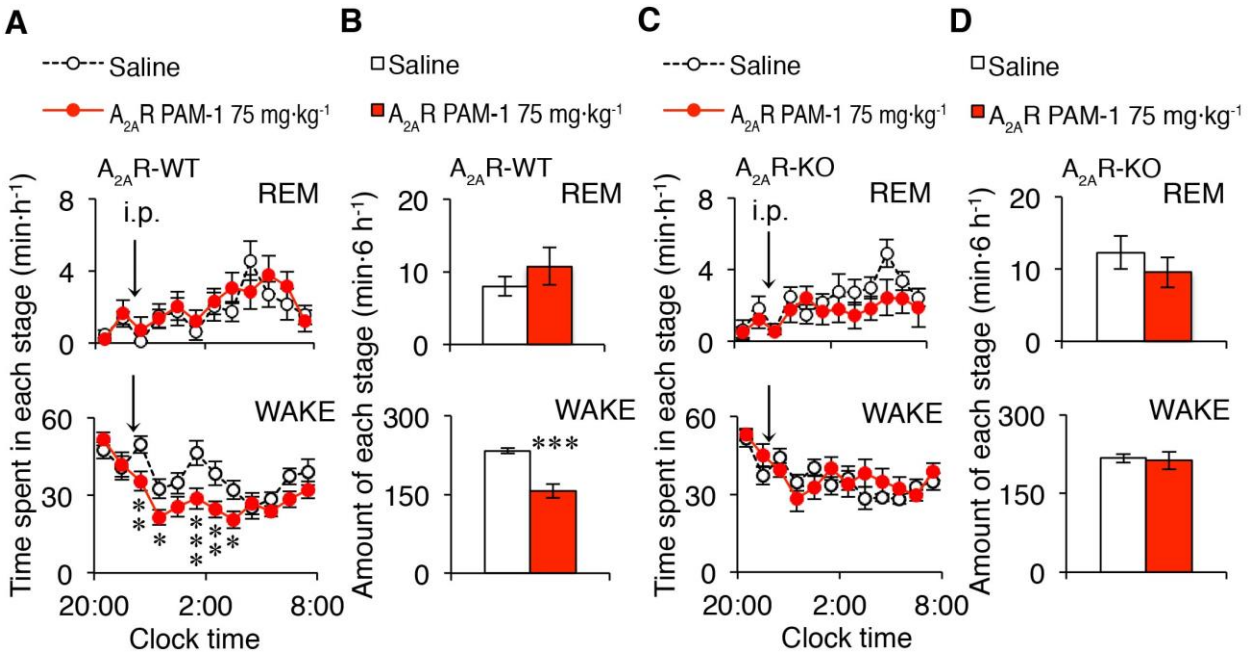

Figure 3.3 REM sleep and wakefulness in wild type and A_{2A}R knockout mice after intraperitoneal administration of A_{2A}R PAM-1.

Time courses (**A** and **C**) and total amount (**B** and **D**) of REM sleep (top panels) and wakefulness (bottom panels) wild type (**A** and **B**) and A_{2A}R knockout mice **(c** and **d)**. Data are presented as the mean ± SEM. *: p<0.05; **: p<0.01; ***: p<0.001,

Compared with saline, assessed by ANOVA Tukey Test or unpaired Student's *t*-test, (n=8/group).

3.4 Summary & Conclusions

Slow-wave sleep-inducing effect of A_{2A}R PAM-1 suppressed by A_{2A}R antagonist, ZM 241385. Furthermore, A_{2A}R PAM-1 activity was abolished in A_{2A}R-KO mice. These findings indicate that SWS inducing effects of A_{2A}R PAM-1 depends on A_{2A}R in mice. Besides that, A_{2A}R PAM-1 did not alter the REM sleep profile of the mice.

CHAPTER 4

Central administration of $A_{2A}R$ PAM-1 effects on slow-wave sleep in mice

4.1 Introduction

Adenosine $A_{2A}R$ is widely expressing in the peripheral system. To confirm that SWS inducing-effect of $A_{2A}R$ PAM-1 is a brain-centric, we infused the $A_{2A}R$ PAM-1 into the lateral ventricular region of the brain via an implanted cannula controlled by the automatic pump system during the dark period. Moreover, the EEG power density of SWS and the sleep architecture of the animals were also analyzed during the infusion of $A_{2A}R$ PAM-1 in the dark period.

4.2 Materials and Methods

4.2.1 Animals

Male mouse lines on a C57BL/6 background, including wild-type and $A_{2A}R$ KO (Chen et al., 1999) mice, which were maintained at the International Institute of Integrative Sleep Medicine and weighing 21-27 g (10-15 weeks old), were used in the experiments. The animals were housed in an insulated and soundproof recording chamber that was maintained at an ambient temperature of 23 ± 0.5°C with a relative humidity of 50 ± 5% and an automatically controlled 12 h light/12 h dark cycle (light on at 8:00, illumination intensity ≈ 100 lux). All animals had free access to food and water. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (2011). Experimental protocols were in compliance with relevant Japanese and institutional laws and guidelines and approved by the University of Tsukuba animal ethics committee (protocol #14-322). Every effort was made to minimize the number of animals used as well as any pain and discomfort experienced by the animals.

4.2.2 Stereotaxic surgery for placement of EEG/EMG electrode and infusion cannula

Mice were anesthetized with pentobarbital [50 $mg \cdot kg^{-1}$, intraperitoneal (i.p.)] and then placed in a stereotaxic apparatus. Electroencephalogram (EEG) and electromyogram (EMG) electrodes for polysomnographic recordings were chronically implanted in the mice (Oishi et al., 2016). The implant comprised two stainless steel screws (1 mm in diameter) inserted through the skull above the cortex (anteroposterior, +1.0 mm; left-right, -1.5 mm from bregma or lambda) according to the atlas of Paxinos and Franklin (Paxinos and Franklin, 2004) that served as the EEG electrodes. Two insulated, stainless steel Teflon-coated wires were placed bilaterally into both trapezius muscles and served as the EMG electrodes. All electrodes. For intracerebroventricular (i.c.v.) infusion of $A_{2A}R$ PAM-1, a stainless-steel cannula was inserted into mice during surgery 0.5 mm anterior and 1.6 mm lateral to bregma to a depth of 1.6 mm below the dura at an angle of 20°, thus placing the cannula into the lateral ventricle. To ensure correct placement of cannula, a plastic tube filled with saline was attached to the infusion cannula; a drop in the meniscus indicated that the cannula tip was in the ventricle. During the experiments, the mice were infused continuously using an infusion pump with artificial cerebrospinal fluid into the lateral ventricle of the brain at a speed of 1 $\mu l \cdot h^{-1}$. Sleep-wakefulness states were monitored for a period of 36 h after infusion of each compound. Saline infusion recordings were obtained in each animal for 36 h, beginning at 20:00, which served as the control for the same animal. In the next experiment, $A_{2A}R$ PAM-1 (200 $nmol \cdot h^{-1}$) was infused into the lateral ventricle of the mouse brain for 12 h (20:00 to 8:00).

4.2.3 Vigilance state assessment by using EEG/EMG polygraphic recordings

Ten days after surgery, the mice were individually housed in transparent barrels in an insulated soundproof recording chamber and connected to the EEG-EMG recording cables for 3 to 5 days of habituation before starting the polygraphic recordings. To evaluate the spontaneous sleep-wake cycle, each animal was recorded for 24 h beginning at 20:00, the onset of the dark period. The animals then entered the pharmacologic phase of the study in which sleep-wakefulness parameters were recorded for 36 h. The data collected during the first 24 h also served as baseline comparison data for the second experimental day. Cortical EEG/EMG recordings were amplified, filtered (EEG 0.5-30 Hz; EMG 20-200 Hz), and digitized at a sampling rate of 128 Hz, and then recording using data acquisition software SleepSign® (Kissei Comtec, Matsumoto, Japan). The vigilance states were classified offline in 10-s epochs into three stages, i.e., wakefulness, rapid eye movement (REM) sleep, and SWS by SleepSign® (ver 3.4) according to standard criteria (Oishi et al., 2016). As a final step, defined vigilance stages were examined visually, and corrected when necessary.

4.2.4 Pharmacological treatment

For control data, mice were infused with saline only (1µl/h) between 20:00-08:00 o'clock. $A_{2A}R$ PAM-1 was dissolved in saline immediately before use and infused into lateral ventricular between 20:00-08:00 o'clock on an experimental day at a concentration of 200 $nmol \cdot \mu l^{-1}$.

4.3 Results

4.3.1 Intracerebroventricular administration of A_{2A}R PAM-1 induces SWS in mice

To elucidate whether the sleep-inducing effect of A_{2A}R PAM-1 is mediated via A_{2A}Rs expressed in the brain, we infused A_{2A}R PAM-1 into the lateral ventricle of wild-type mice at 200 nmol·h^{-1} during the dark period (20:00 to 8:00) and assessed EEG and EMG activity. Infusion with A_{2A}R PAM-1 for 12 h increased the time spent in SWS 5 h after the infusion, resulting in a total SWS increase during the dark period of 141.6 ± 12.5 min compared with saline infusion (**Figure 4.1A, B)**. Concomitantly, total wakefulness was decreased by 145.5 ± 15.8 min during a 12-h i.c.v. infusion of A_{2A}R PAM-1, whereas REM sleep was not affected.

4.3.2 Intracerebroventricular administration of A_{2A}R PAM-1 does not affect EEG power density in SWS.

The EEG activity in the frequency range of 0.5–25 Hz during SWS episodes was indistinguishable between mice treated with saline or A_{2A}R PAM-1 (**Figure 4.2**). These data suggest that A_{2A}R PAM-1 induces physiologic sleep rather than abnormal sleep via A_{2A}Rs that are likely expressed in the brain.

4.3.3 Intracerebroventricular administration of $A_{2A}R$ PAM-1 effects on sleep architecture of mice.

Intracerebroventricular infusion of $A_{2A}R$ PAM-1 (200 nmol·h^{-1}) into mice affected SWS and wake episode numbers during the dark period (**Figure 4.3A**). SWS episode numbers lasting 0 to 29 s, 30 to 59 s, and 60 to 120 s increased by 267%, 196%, and 154%, respectively, and wake episode numbers lasting 0 to 29 s, 30 to 59 s, and 60 to 120 s also increased by 205%, 177%, and 137%, respectively, compared with saline infusion. On the other hand, episode numbers of REM sleep were not significantly affected by $A_{2A}R$ PAM-1 infusion (200 nmol·h^{-1}, i.c.v.). The mean duration of wake episodes decreased by 72% compared with the saline-infused group, but mean episode duration of the SWS and REM sleep did not significantly change after $A_{2A}R$ PAM-1 (200 nmol·h^{-1}, i.c.v.) administration (**Figure 4.3B**). $A_{2A}R$ PAM-1 (200 nmol·h^{-1}, i.c.v.) increased the number of transitions between SWS and wakefulness by 148%, and from wakefulness to SWS by 128% compared with the saline-infused group (**Figure 4.3C**).

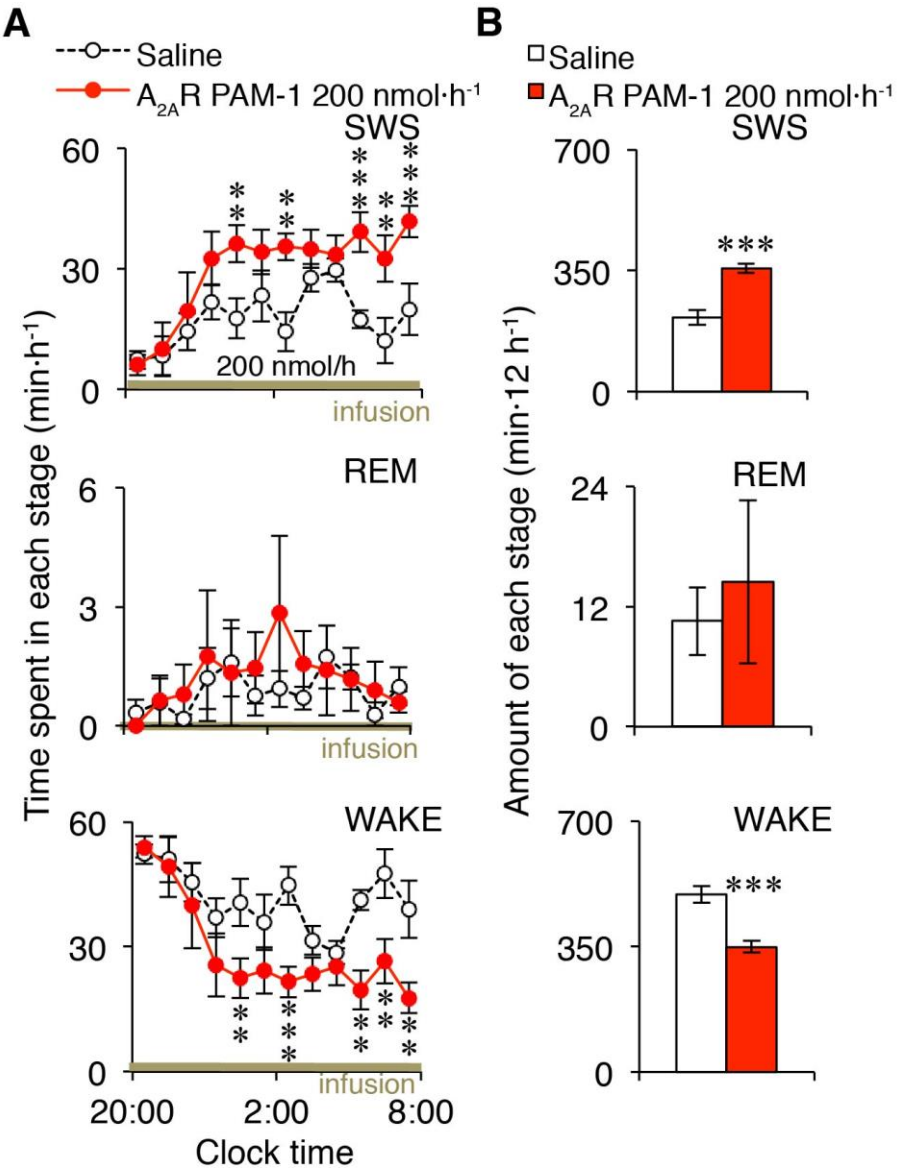

Figure 4.1 Intracerebroventricular infusion of A_{2A}R PAM-1 induced SWS in mice. The time-courses (**A**) and **(B)** total amount of SWS, REM sleep, and wakefulness in mice after intracerebroventricular infusion of saline or A_{2A}R PAM-1. Data are presented as the mean ± SEM. *: p<0.05; **: p<0.01; ***: p<0.001 compared with saline, assessed by ANOVA Tukey Test or Student's *t*-test, (n=5/group).

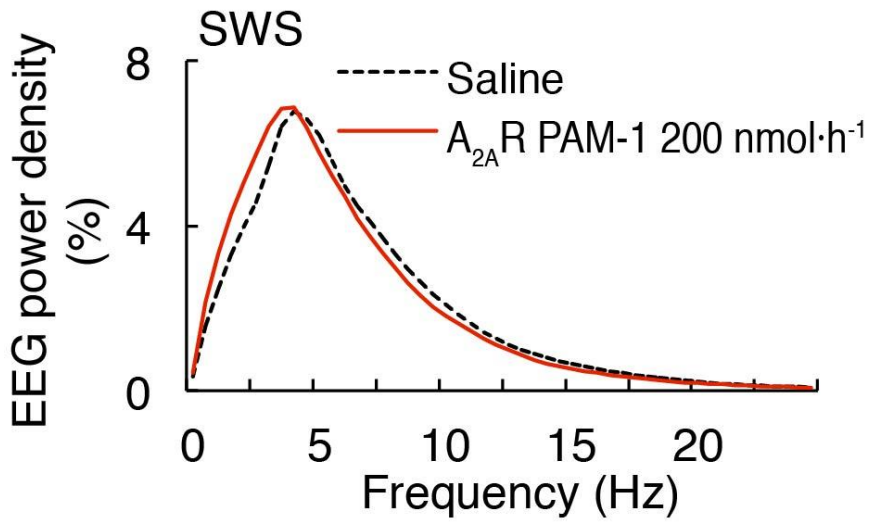

Figure 4.2 EEG power density of SWS during the infusion of saline or A_{2A}R PAM-1. Intracerebroventricular infusion of A_{2A}R PAM-1 does not affect the EEG power density of sleep (n=5/group).

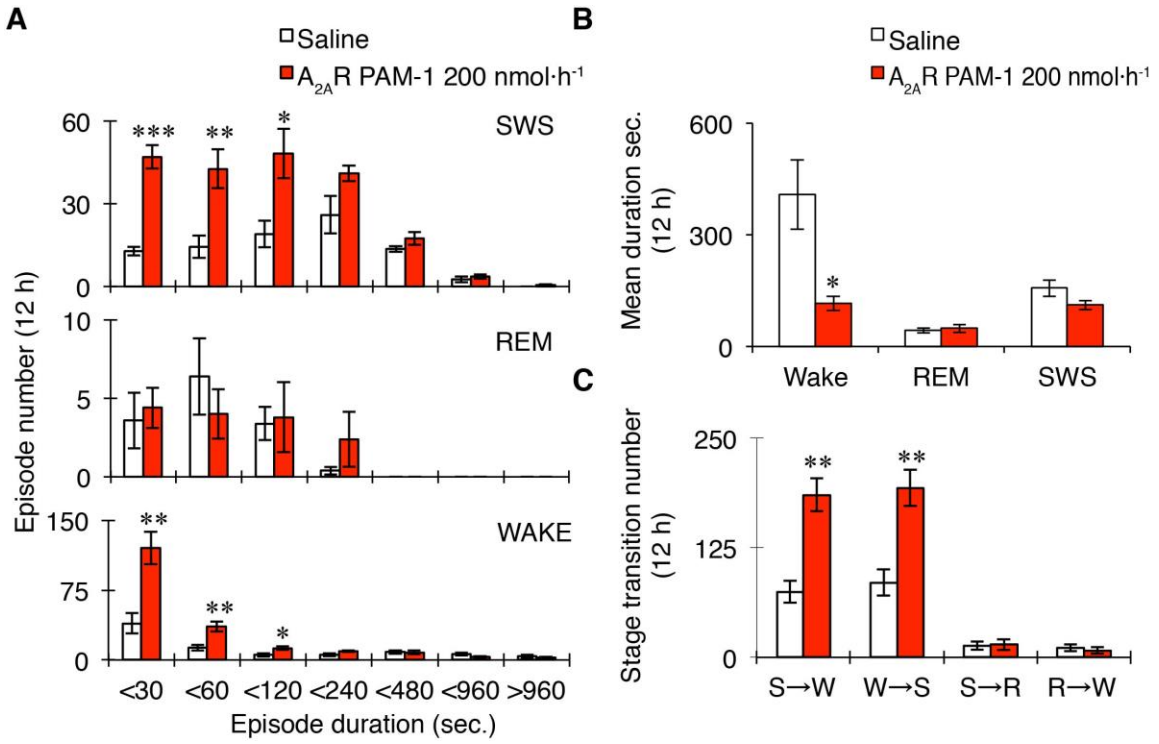

Figure 4.3 Sleep architecture of mice during intracerebroventricular infusion of A_{2A}R PAM-1.

Episode number **(A)** and mean duration **(B)** of each stage after infusion of saline or A_{2A}R PAM-1. (**C**) Transitions between SWS (S), REM sleep (R) and wake (W) stages after infusion of saline or A_{2A}R PAM-1. Data are presented as the mean ± SEM. *: $p<0.05$; **: $p<0.01$, ***: $p<0.001$, compared with saline, assessed by Student's *t*-test, (n=5/group).

4.4 Summary & Conclusions

Intracerebroventricular infusion of $A_{2A}R$ PAM-1 robustly increased the SWS with concomitantly decreasing the wakefulness at the dark period. REM sleep profile of the mice did not affect by the infusion of $A_{2A}R$ PAM-1 at the dark period. In addition, EEG power density of SWS showed an identical rhythm as a control group during the infusion of the $A_{2A}R$ PAM-1. Moreover, $A_{2A}R$ PAM-1 infusion at the dark period increased the SWS episode numbers and decreased the mean duration of the wake stages. It also significantly changed the stage transition number between SWS to wake or wake to SWS.

Chapter 5

The effects of $A_{2A}R$ PAM-1 on body temperature and cardiovascular function

5.1. Introduction

Adenosine A_{2A} receptors are also expressing in cardiovascular systems. There are growing bodies of reports shows that $A_{2A}R$ activation by $A_{2A}R$ agonist causes hypotension. Therefore, we conducted a series of experiments to investigate the effects of the $A_{2A}R$ PAM-1 on the cardiovascular function. After i.p. administration of $A_{2A}R$ PAM-1, we checked the blood pressure of the mice by using the tail-cuff method, heart rhythm of the mice by using intracardiac electrocardiogram and heart rate by using telemetry method. Besides that, we also measured the body temperature of animals after i.p. administration of $A_{2A}R$ PAM-1.

5.2. Materials and Methods

5.2.1 Blood pressure and heart rate measurement

The blood pressure of the mice was measured using the tail-cuff method with a BP-98A blood pressure device (Softron, Tokyo, Japan). The same time period (13:00 – 16:00) was selected for testing the blood pressure of each mouse (9-12 weeks old) to avoid normal daily variations in blood pressure. Five consecutive days were used to habituate the mice to the device. To optimize cardiovascular circulation, mice were wrapped in a cotton sheet and, except for the tail, maintained at 37°C within a cylinder heater. A programmable sensor with an inflatable balloon attached to a tail cuff was used to monitor tail pulse waves and measure blood pressure when the pulse waves were stable and rhythmic. Blood pressure measurement was read and recorded by the software. After five consecutive training days, mice were randomly assigned to one of three groups and injected with saline (10 $ml \cdot kg^{-1}$, i.p.), $A_{2A}R$ PAM-1 (75 $mg \cdot kg^{-1}$, i.p.) or CGS 21680 (1

mg·kg^{-1}, i.p.). Blood pressure was measured at 30 min, 1 h 30 min, and 2 h 30 min after injection (at each time-point, 20 readings for each mouse were collected). After testing, the mice were gently picked up by the tail and gently returned to their cages.

The heart rate of the mice was measured by telemetry. Mice were anesthetized with ketamine hydrochloride (80 mg·kg^{-1}, i.p.) and xylazine hydrochloride (8 mg·kg^{-1}, i.p.) and a PhysioTel F20-ETA mouse telemetry transmitter (Data Science International, St. Paul, MN) was placed in the midline of the mouse back and fixed with surgical sutures. The negative (white) electrode was placed in the trapezius muscle, while the positive (red) electrode was sutured to a muscle in the back opposite the xiphoid process. Each mouse was singly housed in a cage after surgery with a distance of at least 1 m between cages to avoid interference between telemetry transmitters. After 7 days of recovery, the mice were randomly assigned to one of three groups and injected with saline (10 ml·kg^{-1}, i.p.), $A_{2A}R$ PAM-1 (75 mg·kg^{-1}, i.p.), or CGS 21680 (1 mg·kg^{-1}, i.p.). The transmitted cardiovascular signal was analyzed for 2 h after the injections using Data Science International software.

5.2.2 Heart rhythm measurement

The cardiac rhythm of mice was measured by electrocardiography (ECG). Mice were anesthetized with ketamine hydrochloride (80 mg·kg^{-1}, i.p.) and xylazine hydrochloride (8 mg·kg^{-1}, i.p.) and fixed with needles on a styrofoam platform. Mice were then gently pushed into a position where the two front paws and the left rear paw are in contact with 25-gauge needles that served as ECG electrodes. For intracardiac electrography, the

throat of the mice was opened and the internal jugular vein was isolated to insert a catheter along the course of the vein to the right atrium. Electrographic signals were 5.000-10.000-fold amplified and filtered (0.5-250 Hz) with an AC-601G system (Nihon Kohden, Tokyo, Japan). The same time period (10:00 – 12:00) was selected for testing the heart rhythm to avoid normal daily variations in the cardiac rhythm. Mice were randomly assigned to groups that received $A_{2A}R$ PAM-1 (75 $mg \cdot kg^{-1}$, i.p.) or CGS 21680 (1 $mg \cdot kg^{-1}$, i.p.) injections. After recording the baseline for 1-2 minutes, mice were injected with drugs and recording continued for 30 minutes. The data were analyzed using LabChart Pro software (ADInstruments, Dunedin, New Zealand).

5.2.3 Body temperature measurement

The core body temperature of mice was measured using Thermochron iButtons (KN Laboratories, Osaka, Japan). iButtons were programmed to monitor core body temperature every 5 min for 14 consecutive days beginning at the end of the recovery period. The mice were anesthetized with pentobarbital (50 mg·kg-1, i.p.), and the skin of the abdomen was shaved and cleaned with 70% ethanol. A longitudinal, 2-cm incision was made along the midline. One iButton cleaned with 70% ethanol was placed in the abdominal cavity and the incision was closed with nylon sutures. The mice were housed individually in cages after surgery under 12:12 light/dark cycles and experiments were conducted after a 10-day recovery period. iButtons were removed from the animals after cervical dislocation euthanasia and RhManager software (KN Laboratories, Osaka, Japan) was used to collect the recorded data from the iButtons.

5.3 Results

5.3.1 Intraperitoneal administration of $A_{2A}R$ PAM-1 does not affect blood pressure and hear rate in mice

$A_{2A}R$ agonists evoke cardiovascular effects (Hutchison et al., 1989; Kirkup et al., 1998; Nekooeian and Tabrizchi, 1996). We therefore tested the effect of intraperitoneal administration of $A_{2A}R$ PAM-1 on blood pressure and heart rate in wild-type mice. First, we measured blood pressure in mice 30, 90, and 150 min after intraperitoneal injection of 75 $mg \cdot kg^{-1}$ $A_{2A}R$ PAM-1 or 1 $mg \cdot kg^{-1}$ of the $A_{2A}R$ agonist CGS 21680 using an electrosphygmomanometer (**Figure 5.1)**. The dose of the $A_{2A}R$ agonist CGS 21680 was selected based on previous studies in mice (Carvalho et al., 2017; Nakav et al., 2008; Ohta and Sitkovsky, 2001). Compared with saline treatment, the systolic, and diastolic blood pressures were significantly decreased for up to 90 min after injecting the $A_{2A}R$ agonist CGS 21680 and returned to normal levels within 150 min after the injection. In contrast, blood pressure was not changed after intraperitoneal administration of $A_{2A}R$ PAM-1 (75 $mg \cdot kg^{-1}$) at 30, 90, or 150 min after treatment. In addition, we measured the heart rate of mice after intraperitoneal injection of 75 $mg \cdot kg^{-1}$ $A_{2A}R$ PAM-1 or 1 $mg \cdot kg^{-1}$ $A_{2A}R$ agonist CGS 21680 using the telemetry implants (**Figure 5.2**). The heart rate of the mice increased after intraperitoneal administration of the $A_{2A}R$ agonist CGS 21680, whereas the heart rate was not affected by injection of 75 $mg \cdot kg^{-1}$ $A_{2A}R$ PAM-1.

5.3.2 Intraperitoneal administration of $A_{2A}R$ PAM-1 does not affect heart rhythm in mice

We monitored the heart rhythm in anesthetized mice after intraperitoneal administration of 75 $mg \cdot kg^{-1}$ of $A_{2A}R$ PAM-1 or 1 $mg \cdot kg^{-1}$ of $A_{2A}R$ agonist CGS 21680 using intracardiac EGM. We observed sinus arrhythmia in mice after intraperitoneal administration of $A_{2A}R$ agonist CGS 21680, whereas injection of 75 $mg \cdot kg^{-1}$ $A_{2A}R$ PAM-1 did not cause abnormalities of the cardiac rhythm (**Figure 5.3A-C**).

5.3.2 Intraperitoneal administration of $A_{2A}R$ PAM-1 does not affect body temperature in mice

We also measured the effect of intraperitoneal administration of 75 $mg \cdot kg^{-1}$ $A_{2A}R$ PAM-1 or 1 $mg \cdot kg^{-1}$ of the $A_{2A}R$ agonist CGS 21680 (as positive control) on the body temperature of mice during the dark period (**Figure 5.4**). Whereas CGS 21680 strongly decreased the body temperature for almost 2 h ($t_{(10)}$=3.68, P=0.0042 at 22:15, $t_{(10)}$=10.48, P<0.0001 at 23:15, $t_{(10)}$=2.33, P=0.041 at 00:05 vs. saline injected group, unpaired t-test), $A_{2A}R$ PAM-1 did not affect the body temperature of the mice. These data suggest that $A_{2A}R$ PAM-1 induces physiological sleep independent of the body temperature.

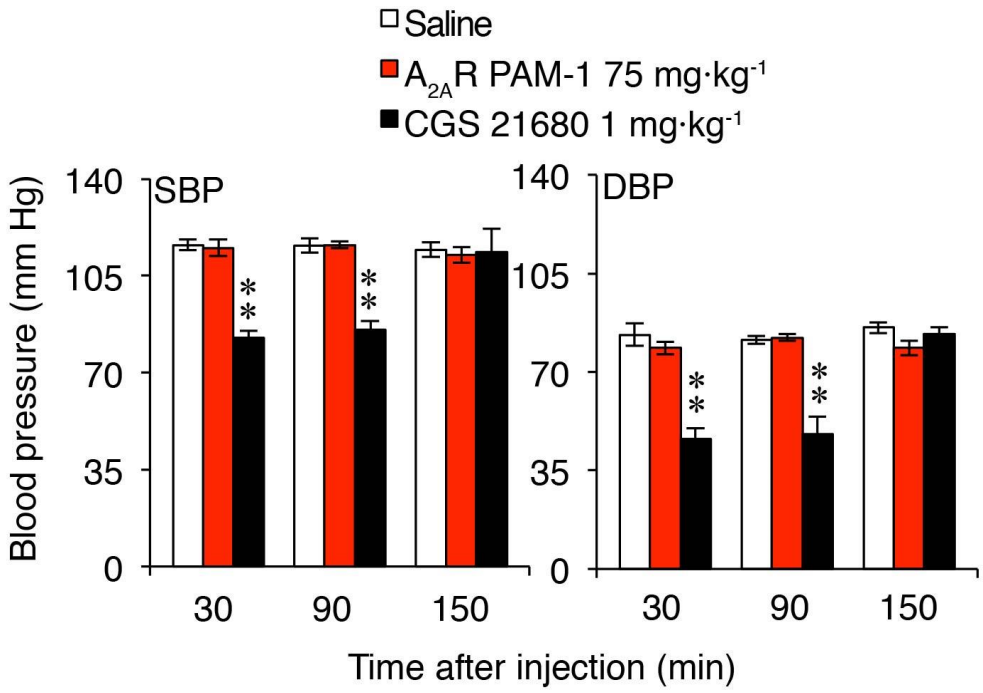

Figure 5.1 A_{2A}R PAM-1 does not affect blood pressure in mice.

Blood pressure after A_{2A}R PAM-1 and CGS 21680 injection in mice. Data are presented as the mean ± SEM. **: $p<0.01$ vs. saline, Student's *t*-test. (n=6/group). Abbreviations used: CGS 21680, selective adenosine A_{2A} receptor agonist; SBP, systolic blood pressure; DBP, Diastolic blood pressure.

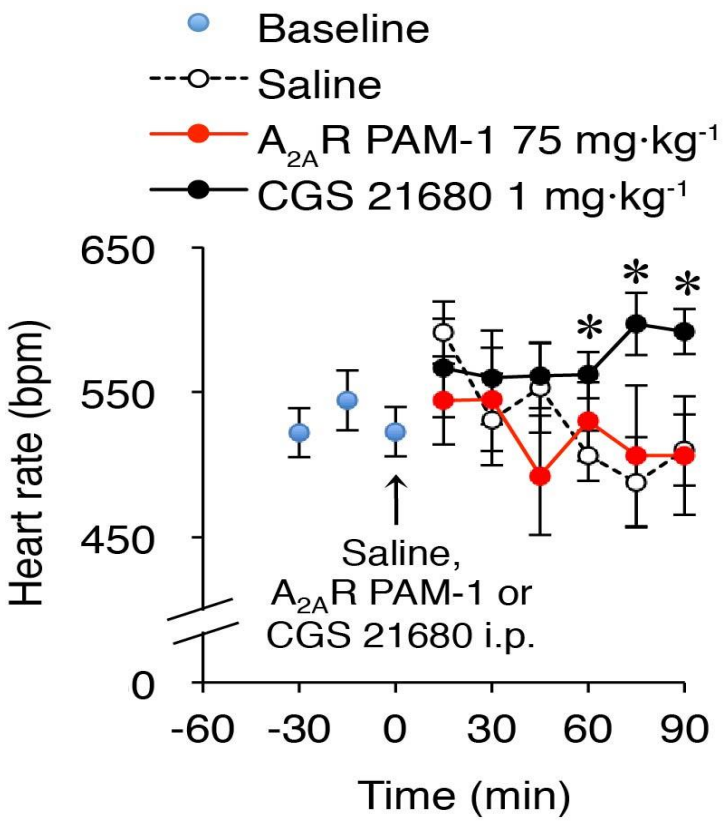

Figure 5.2 A_{2A}R PAM-1 does not affect heart rate in mice

Heart rate of mice after injection of saline, A_{2A}R PAM-1, or CGS 21680, assessed by the telemetry implants. Data are presented as the mean ±SEM. *: $p<0.05$ vs. Saline, Student's *t*-test. (n=5/group). CGS 21680, selective adenosine A_{2A} receptor agonist.

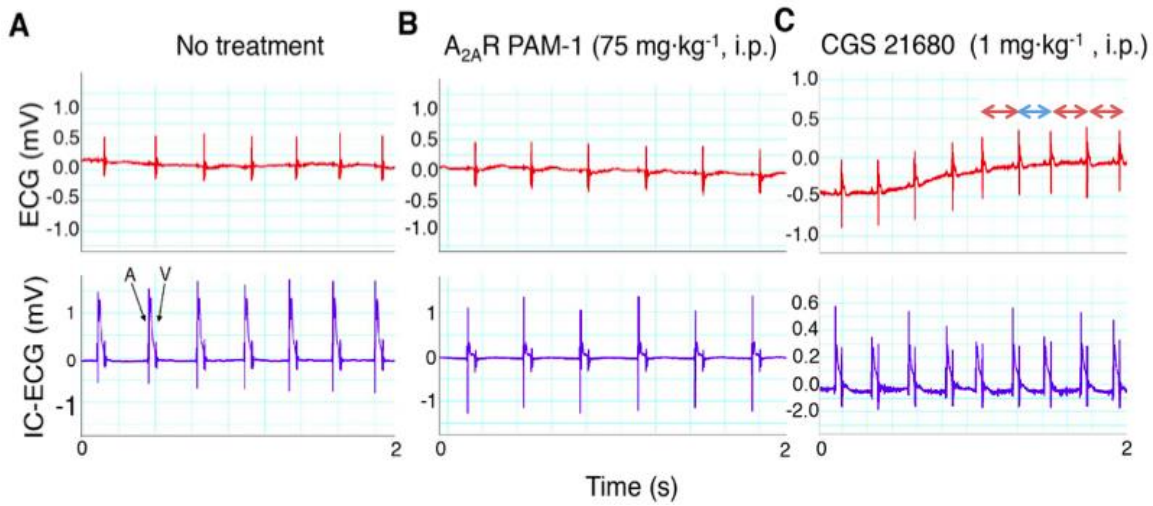

Figure 5.3 A_{2A}R PAM-1 does not affect heart rhythm in mice.

Typical heart rhythm profiles of mice without treatment (**A**) or after administration of A_{2A}R PAM-1 (**B**) or CGS 21680 (**C**). Red and blue left/right arrows in the right panel indicate sinus arrhythmia.

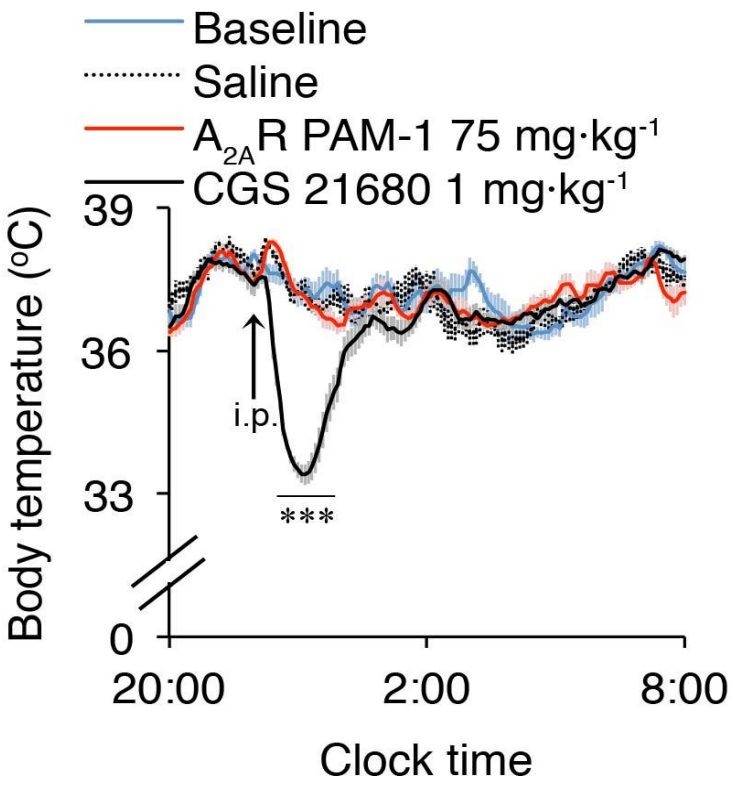

Figure 5.4 A_{2A}R PAM-1 does not affect body temperature in mice.

Body temperature of mice after intraperitoneal administration of saline, A_{2A}R PAM-1 or CGS 21680. Data are presented as the mean ±SEM. **: $p<0.01$ vs. Saline, Student's *t*-test. (n=6/group).

5.4 Summary & Conclusions

As like we expected, $A_{2A}R$ potent agonist CGS 21680 was significantly decreased the blood pressure of the mice during two hours after injection and it also caused irregular heart rhythm or sinus arrhythmia. On the other hand, $A_{2A}R$ PAM-1 did not alter the blood pressure and heart rhythm of the mice compared to the control groups. Moreover, CGS 21680 also significantly decreased the heart rate for two hours after injection but $A_{2A}R$ PAM-1 did not cause any significant change during this time period. Besides that, whereas CGS 21680 strongly decreased body temperature for almost 2 hours, $A_{2A}R$ PAM-1 did not affect the body temperature of the mice. These data suggest that $A_{2A}R$ PAM-1 induces physiological sleep independent of the body temperature.

General Discussions

Our observations suggest that enhancing $A_{2A}R$ signaling by intraperitoneal administration of $A_{2A}R$ PAM-1 induces SWS without cardiovascular effects in mice. Therefore, $A_{2A}R$-modulating compounds may provide safe options for the treatment of insomnia and poor-quality sleep.

Over the past century, several putative hypnogenic substances implicated in the sleep homeostatic process have been identified, including prostaglandin D_2 (Qu et al., 2006b), cytokines (Krueger et al., 1984b), anandamide (García-García et al., 2009b), urotensin II peptide (Huitron-Resendiz et al., 2005), and adenosine (Porkka-Heiskanen et al., 1997). Adenosine represents a state of relative energy deficiency: ATP depletion positively correlates with an increase in extracellular adenosine levels (Kalinchuk et al., 2003) and positively associates with sleep (Porkka-Heiskanen et al., 1997). Adenosine levels in samples collected from several brain areas of cats during spontaneous sleep-wake cycles by *in vivo* microdialysis were higher during SWS than during wakefulness for all probed brain areas (Porkka-Heiskanen et al., 1997). The observation in animals that adenosine levels are elevated during prolonged wakefulness may explain why an allosteric modulator could effectively enhance the sleep-inducing effect of endogenous adenosine in the brain. On the other hand, adenosine is absent or its concentration is too low in the cardiovascular system under physiologic conditions to affect blood pressure and heart function after administration of an allosteric modulator of $A_{2A}R$.

Medicinal chemistry for $A_{2A}Rs$ has been widely developed in recent decades for use in myocardial perfusion imaging and the treatment of inflammation and neuropathic pain

(de Lera Ruiz et al., 2014). Several $A_{2A}R$ agonists that entered clinical trials elicited undesirable side effects, however, thus precluding their further development. On the other hand, allosteric modulators bind at a distinct site other than the natural ligand binding site (i.e., the orthosteric site) and exert their effects only in the presence of the orthosteric ligand (Wenthur et al., 2014). As a consequence, an allosteric modulator mimics the activity duration of the natural ligand and thus the pharmacologic response of an allosteric modulator more closely resembles the natural physiologic activity of the receptor than is possible with a synthetic agonist. Because efforts to evoke pharmacologic $A_{2A}R$ responses have focused almost exclusively on the use of orthosteric ligands, however, the possibility that $A_{2A}R$ responses, especially in the brain, can be fine-tuned using allosteric modulators has received very little attention (Göblyös and Ijzerman, 2009).

Moreover, it is widely accepted that the basic adenosine scaffold must be maintained in an $A_{2A}R$ agonist (Fredholm et al., 2011). Thus, the development of adenosine analogs for treating the central nervous system, including sleep induction for treating insomnia, is restricted by the poor transport of these drugs through the brain endothelial cells, which are connected by tight junctions to establish a blood-brain barrier (BBB) (Pardridge et al., 1994). In contrast, $A_{2A}R$ PAM-1, when administered intraperitoneally, exhibits a sleep-inducing effect that is likely mediated by $A_{2A}Rs$ in the brain and thus appears to cross the BBB. Small lipophilic monocarboxylates like $A_{2A}R$ PAM-1 likely pass through the BBB by passive diffusion or via a monocarboxylate transport system (Tsuji, 2005). Therefore,

allosteric modulation of A_{2A}Rs has the potential to cause pharmacologic effects in the central nervous system after systemic administration, resulting in good quality sleep.

Our study did not investigate how and where the A_{2A}R PAM-1 binds at the receptor to exert its allosteric effect. Therefore, an important next step will be to examine allosteric interactions of A_{2A}R PAM-1 and the receptor using binding assays and crystal structure analysis. With respect to the latter, the crystal structure of the human A_{2A}R bound to a bitopic antagonist revealed a potential allosteric pocket (Sun et al., 2017) and another study suggested that a sodium ion binding site could be exploited for allosteric modulation of A_{2A}R (Gutiérrez-de-Terán et al., 2013). Moreover, to solidify the sleep enhancing effect of the A_{2A}R PAM-1, it may be necessary to test the A_{2A}R PAM-1 in mice at the time of normal sleep onset, i.e., A_{2A}R PAM-1 administration at the onset of the light period, or in an animal model of insomnia, for example, a mouse model mimicking the human first-night effect (Xu et al., 2014).

Due to work schedules and expectations, lifestyle choices, pre-existing medical conditions, or aging, people are coping with an increasingly wide range of sleep problems, including difficulties with falling and staying asleep, waking up too early, and poor-quality ("non-restorative") sleep. Deficiencies in sleep cause significant social losses due to increased prevalence of mood disorders, lead to decreased economic productivity, and are linked to traffic and work-related accidents due to excessive daytime sleepiness (Groeger et al., 2004; Saddichha, 2010; Sutton et al., 2001). Insufficient sleep is not only by itself a major problem in modern society, but is also an

established risk factor for obesity, diabetes, heart disease, and other lifestyle diseases (Colten et al., 2006). Moreover, psychiatric illnesses, especially anxiety and mood disorders, are long recognized to be a frequent cause of insomnia (Okuji et al., 2002).

Conclusions

The findings of our study indicate that enhancing $A_{2A}R$ signaling promotes SWS without cardiovascular effects. Therefore, small molecules that allosterically modulate $A_{2A}Rs$ could help people with sleep problems to fall asleep and thus also be a potential treatment for psychiatric disorders.

Our study was conducted in mice which are the most commonly used model organism in human disease. The mouse is, however, not particularly reliable at predicting the outcome of studies in humans, mostly due to limited genetic diversity associated with common laboratory mice. Therefore, many obstacles in generating a novel drug for the treatment of insomnia in humans remain.

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